# Optimizing Mycobacterium tuberculosis detection in resource limited settings



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**Optimizing Mycobacterium tuberculosis detection in resource limited settings**

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**Abstract**

**Objectives:** The light-emitting diode (LED) florescence microscopy has made acid-fast bacilli (AFB) detection faster and efficient although its optimal performance in resource limited settings is still being studied. We assessed the optimal performances of light and florescence microscopy in routine conditions of a resource limited setting and evaluated the digestion time for sputum samples for maximum yield of positive cultures.

**Design:** Cross-sectional study

**Setting:** Facility-based involving samples of patients receiving tuberculosis treatment and care from the main tuberculosis case referral center in northern Nigeria.

**Participants:** The study included 450 sputum samples from 150 new patients with clinical diagnosis of pulmonary tuberculosis.

**Methods:** The 450 samples were pooled into 150 specimens, examined independently with mercury vapor lamp (FM), LED CyScope® (CY) and Primo Star iLED (PiLED) florescence microscopies, and with the Ziehl Neelsen (ZN) microscopy to assess the performance of each technique compared to liquid culture. The cultured specimens were decontaminated with NaOH-NaCL for 10, 15 and 20 minutes before incubation in Mycobacterium Growth Incubator Tube (MGIT) system and growth examined for acid-fast bacilli (AFB)

**Results:** Of the 150 specimens examined by direct microscopy: 44 (29%), 60 (40%), 49 (33%) and 64 (43%) were AFB positive by ZN, FM, CY, and iLED microscopies. Digestion of sputum samples for 15min yielded the highest number of AFBs as illustrated by AFB growth in 72 (48%), 81 (54%) and 68 (45%) cultures with sputum samples decontaminated at 10, 15 and 20 minutes respectively.

**Conclusions:** In routine laboratory conditions in resource-limited setting, our study has demonstrated the superiority of the florescence and the Primo Star iLED microscopy. Digestion of sputum samples for 15minutes yielded more positive cultures

**Keywords:** Tuberculosis, MICROBIOLOGY, Respiratory infections

**Article focus**:

1. What is the optimal performance of the new light-emitting diode (LED) florescence microscopy in routine conditions of a resource limited setting and how does it compare to the traditional light microscopy using Ziehl Neelsen (ZN) technique for AFB smear microscopy?
2. What is the optimal digestion time for sputum samples for maximum yield of positive cultures in routine conditions of a resource limited setting?
3. Despite the operational challenges of resource limited settings florescence microscopy has better optimal performance compared to the traditional light microscopy using Ziehl Neelsen (ZN) technique for AFB smear microscopy.

**Significance**:

1. Most of the settings with highest burden of Tuberculosis in the world are limited in resource needed to effectively detect and manage cases of the disease.
2. The WHO has recommended the phasing out of the simple, inexpensive but less sensitive ZN technique with the more sensitive florescence technique for better TB detection in all settings.
3. Evaluating the performance of the more sensitive florescence microscopy against the less sensitive ZN microscopy in unaltered working conditions of resource limited settings is relevant for effective TB control.

**Strength and Limitations**:

1. The design of this study allows test performance assessment under routine (real) resource constrain settings.
2. Blinding the microscopist on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of direct smear results
3. However, our inability to obtain participant HIV status deprived us the ability to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures.

## Introduction

Globally, an estimated 1.4 million death occurred in 2011 as a result of infection with tuberculosis, one-fourth of the death was associated with HIV infection and most of it in resource limited settings (1) where the burden of HIV infection is high. In Africa, TB is the leading cause of death among HIV infected persons as the continent harbors eighty percent of the world HIV-TB cases (2). Nigeria and the Republic of South Africa are among the top five TB burden nations of the world. Sensitive, specific, and inexpensive point-of-care diagnostic tools for rapid TB detection are lacking posing challenges to the optimal diagnosis of TB in resource limited settings (RLS), particularly among persons with HIV infections whose sputum specimen often lack sufficient quantity of acid-fast bacilli (AFB) detectable on routine smear examination (3, 4)

The simplicity, inexpensiveness and predictive power of the Ziehl Neelsen sputum smear microscopy make it the applicable laboratory diagnostic tool of choice for tuberculosis in resource limited settings. (5) When properly done, a positive sputum-smear is highly predictive of active TB (6, 7). Unfortunately, its sensitivity is low and performance is often affected by lack of proper maintenance of equipment; trained manpower; and quality assurance system in settings where resources are scarce. (8) Even in centers with good quality assurance measures, smear AFB detects only 40-50% of TB cases compared to about 80% yield by culture (9). However, it remains the only available tool for bacteriologic diagnosis of TB in most RLS. Culture techniques are highly sensitive and specific, but the cost, technical complexity and time delay before results are produced make culture unscalable for rapid detection and treatment of tuberculosis. Recent advances have made it possible to use molecular technology to detect AFBs with great accuracy. However these technologies still require a fairly developed laboratory with a reliable source of electricity.

Detection of smear positive cases is the priority in TB control programs. These cases are infectious and contribute substantially to the transmission of the disease. (10, 11) In recent years, several new laboratory techniques have been developed to significantly improve the ability to diagnose active TB in populations. Fluorescence microscopy improves the sensitivity of sputum-smear detection and has good specificity relative to the ZN method. Inexpensive light-emitting diode-based (LED) fluorescence microscopes have been developed that make microscopy of smears stained by Auramine florescence dye easier, cheaper and faster with potentials for scalability to remote clinics in RLS. (5, 12-14) And, they can be battery operated as well.

This study compared the performance of the standard but more expensive mercury vapor lamp (FM) florescence microscope; the newer, less expensive LED based CyScope® (CY) (Partec, Görlitz, Germany); and the Primo Star iLED (PiLED) (Carl Zeiss Inc. Germany) florescence microscopes in comparison to the conventional light ( ZN) microscope against the gold (reference) standard of liquid culture in automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems) for the detection of AFB from sputum samples of patients with clinical pulmonary tuberculosis (TB). In addition, we evaluated the optimal processing conditions for liquid culture in the study population.

## Methods

*Study population*

Three sputum samples (spot-morning-spot) were collected from 150 TB treatment naïve, consecutive patients with clinical diagnosis of pulmonary tuberculosis at the National TB and Leprosy Training Center (NTBLTC), Zaria, Nigeria. The spot-morning-spot sample collection was the standard routine for the diagnosis of TB in Nigeria at the time of the study. This study was conducted within the routine laboratory protocols for TB control and the investigators had no direct contact with the patients. Samples were completely de-identified after the initial results were obtained for clinical care and no additional clinical data were collected. This study was conducted from July to September of 2009.

*Smear Microscopy*

The three sputum samples from each patient were pooled. Four direct smears were made from each of the pooled sputum samples with applicator sticks on clean grease-free slides; measuring 2 cm x 3 cm, and not too thick. The first slide was stained with Ziehl Neelsen (ZN) stain according to the WHO recommended protocol described elsewhere (15). Briefly, the smear was air dried and fixed by gently passing it over a flame 2-3 times. One percent strong carbol fuchsin was applied to the slide and heated with a Bunsen flame intermittently 3 times and allowed to stain for 15 minutes. The stain was rinsed off with gentle tap water and 3% acid alcohol was applied on the smear for 3 minutes. Methylene blue (0.3%) was then added for up to 1 minute and rinsed off with tap water. Positive and negative controls were included in the process. Examination of the slide was done with light microscope at 1,000x magnification and the AFBs identified were graded.

The second, third and fourth slides were air-dried, heat-fixed and stained with Auramine O-phenol for 15 minutes rinsed with tap water, decolorized with 1 percent acid alcohol for 2 minutes then rinsed with water and counterstained with 0.1 percent potassium permanganate for 2 minutes. The slides were rinsed with water then allowed to drain dry in air before examination under FM, CY and PiLED microscopes respectively. The AFBs were visualized and graded at magnifications of 200× and 400× according to a protocol describe by Lehman (16). A total of 100 microscopic fields were examined before a slide is recorded as positive (if at least an AFB was seen) or negative (if no AFB was seen). Positive smears were graded as scanty (actual) if there were: 1-9 AFBs in at least 100 fields; +1: 10-99 in 100 fields; +2: 1-10 per field in at least 50 fields; and +3: if there were more than 10 per field in at least 20 fields. Absence of any AFB was graded as negative smear. Each slide was examined by three independent readers to ascertain the presence of AFB and grade positive smears. The slide readers were blinded on the clinical or laboratory diagnosis of the subjects whose samples were examined.

*Specimen culture*

Each of the 150 pooled sputum specimens were decontaminated with BD Mycoprep$^{TM}$ (Beckton Dickinson Diagnostic Systems, Sparks, Maryland, USA) which consists of 1% N-acetyl-L-cysteine (NALC), 4% sodium hydroxide and 2.9% sodium citrate. An equal amount of Mycoprep was added to the sputum, homogenized and allowed to act for 10, 15 and 20 minutes respectively. After the respective digestion period, phosphate buffered saline was added to stop the digestion and decontamination reaction. This was then centrifuged under refrigerated conditions with uniform centrifugation time of 15 minutes and a Relative Centrifugal force (RCF) of 3000 x g. The supernatants were discarded and the sediments re-suspended with 1 ml of phosphate buffered saline to make a final volume of 2 ml. Then 0.5 ml of each was inoculated into Mycobacterium Growth Indicator Tubes (MGIT) and incubated in the automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems), which monitors growth.

The cultures that indicated positive growth were removed from the machine and the presence of AFB confirmed by ZN smear microscopy. Those that do not indicate growth after 42 days were classified as negatives as recommended by the manufacturer and as was the standard practice. All cultures were done at Zankli Research laboratory while all microscopy were performed at the NTBLTC, Zaria in order not to influence the outcome of culture or direct microscopy results.

*Statistical analysis*

The raw data generated from the smear microscopic examinations of the 150 matched samples by the four different microscopes as well as the data generated after same samples were cultured following decontaminations at 10, 15 and 20 minutes were organized into cell counts in which descriptive values were provided along with frequencies and proportions. Measures of accuracy for the different smear examinations: sensitivity, specificity, positive and negative predictive values were evaluated through decision matrix tables while concordance and disagreements between the microscopic examination by each of the three florescence microscopes and the conventional light (ZN) microscope based on the final classification of samples by the gold standards (outcomes MGIT cultures) were examined matched sample tables for positive and negative final outcomes as previously recommended for comparative analysis of diagnostic procedures performed on same samples (patients) (17). Comparison between the florescence microscopies to the conventional ZN microscopy for differences in performance together with their 95% confidence intervals was done using the McNemar's test. Significance of association was set at 0.05 alpha and all probabilities were two tailed.

## Results

Of the 150 matched (paired) samples, AFBs were detected in: 44 (29%) of the ZN smears examined under light microscope; 60 (40%) of the AR smears examined with FM; 49 (33%) of the AR smears examined with CY; and 64 (44%) AR smears examined with PiLED florescence microscopes. MGIT cultures of the 150 specimen yielded 72 (48%) AFB positives in the group decontaminated for 10 minutes (A) prior to incubation; 81 (54%) in those decontaminated for 15 minutes (B) and 68 (45%)in those with 20 minutes (C) decontamination time. Of the direct microscopy techniques, AR smears examined by the PilED gave the highest yield and was surprising comparable to cultures from 10 and 20 minutes digestion. As expected the ZN detected the list number of positive slide so did the CY.

Among the positive AFB tests graded, ZN light microscopy produced the highest proportion of samples with scanty AFBs on examination 21/44 (48%), followed by PiLED florescence microscopy 12/64 (19%), then CY 8/49 (16%) and FM had the least scanty AFB positive results 3/60 (5%). However when the samples with scanty positive AFBs where incubated in A, B and C MGIT culture groups, the proportions confirmed by the cultures in the sequence above were as follows: (i) ZN: 14/21 (67%), 9/21 (42%) and 11/21 (52%); (ii) PiLED: 5/12 (42%), 7/12 (58%) and 5/12 (42%); (iii) CY: 6/8 (75%), 4/8 (50%) and 3/8 (38%); lastly (iv) FM: 0/3 (0%), 1/3 (33%) and 1/3 (33%). A summary of the positive AFB outcomes from the direct smear examinations by microcopy types, grades and the proportions of graded smears confirmed by the MGIT cultures were provided in Table 1.

Table 1. Proportions of positive graded direct smears confirmed by cultured specimen decontaminated at 10 (A), 15 (B) and 20(C) minutes obtained from 150 matched samples

| Direct smear (DS) | Grade | Frequency | MGIT cultured specimen (Gold standards) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A | | B | | C | |
| | | | n | % | n | % | n | % |
| ZN | scanty | 21 | 14 | 66.7 | 9 | 42.1 | 11 | 52.4 |
| | +1 | 13 | 12 | 92.3 | 11 | 84.6 | 10 | 76.9 |
| | +2 | 5 | 5 | 100.0 | 4 | 80.0 | 5 | 100.0 |
| | +3 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| FM | Scanty | 3 | 0 | 0.0 | 1 | 33.3 | 1 | 33.3 |
| | +1 | 34 | 24 | 70.6 | 19 | 55.9 | 17 | 50.0 |
| | +2 | 18 | 16 | 88.9 | 12 | 66.7 | 13 | 72.2 |
| | +3 | 5 | 4 | 80.0 | 4 | 80.0 | 4 | 80.0 |
| CY | Scanty | 8 | 6 | 75.0 | 4 | 50.0 | 3 | 37.5 |
| | +1 | 30 | 22 | 73.3 | 18 | 60.0 | 19 | 63.3 |
| | +2 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| | +3 | 6 | 6 | 100.0 | 6 | 100.0 | 6 | 100.0 |
| PiLED | Scanty | 12 | 5 | 41.7 | 7 | 58.3 | 5 | 41.7 |
| | +1 | 36 | 22 | 61.1 | 20 | 55.6 | 18 | 50.0 |
| | +2 | 13 | 13 | 100.0 | 10 | 76.9 | 13 | 100.0 |
| | +3 | 3 | 3 | 100.0 | 2 | 66.7 | 3 | 100.0 |

ZN: Ziehl Neelsen microscopy using the conventional light microscope
FM: Florescence microscopy with mercury vapor lamp microscope
CY: Florescence microscopy with Cyscope light emitting diode microscope
PiLED: Florescence microscopy with Primo Star iLED light emitting diode microscope

A comparison of the outcomes of direct smear tests with the final culture results to measure the performances of the direct smear techniques against the gold standards showed near identical frequency distributions for the true positive (TP), false positive (FP), false negative (FN) and true negative (TN) for the ZN and CY microscopy techniques. The FM microscopy however yielded a pattern of distribution similar to the PiLED techniques as shown in the decision matrix of Table 2.

Table 2. Decision matrix for the evaluation of performance of the direct smear methods against the different gold standards for the 150 matched samples

| Direct Smear (DS) | DS Outcome | Gold standards: MGIT cultures | | | | | |
|---|---|---|---|---|---|---|---|
| | | A : culture positive =72 | | B: culture positive =81 | | C: culture positive = 68 | |
| | | P | N | P | N | P | N |
| ZN | P | 36 | 08 | 29 | 15 | 31 | 13 |
| DS+ = 44 | N | 36 | 70 | 52 | 54 | 37 | 69 |
| FM | P | 44 | 16 | 36 | 24 | 35 | 25 |
| DS+ =60 | N | 28 | 62 | 45 | 45 | 33 | 57 |
| CY | P | 39 | 10 | 33 | 16 | 33 | 16 |
| DS+ =49 | N | 33 | 68 | 48 | 53 | 35 | 66 |
| PiLED | P | 43 | 21 | 39 | 25 | 39 | 25 |
| DS+ =64 | N | 29 | 57 | 42 | 44 | 29 | 57 |

Culture specimen decontaminated by NaOH-NaCL : A for 10 minutes; B for 15minutes; C for 20 minutes

DS+ = number of samples (out of 150 samples) that were AFB positive by direct smear

P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor florescence microscopy; CY=CyScope florescence microscopy and PiLED= Primo Star iLED florescence microscopy

The levels of agreement and differences between the convention ZN microscopy and each of the florescence microscopy techniques with respect to the final culture results were described by the concordance and discordance cells displayed in Table 3. Cells were discordant if the outcomes of the two techniques disagreed otherwise they were concordant. The statistical significance of the difference in the performance of the techniques compared was derived from the discordant cell. The sum of the frequencies of the discordant cells for example that explained the differences between ZN and CY were 7, 4 and 2 for the positive culture results which showed less consistent divergence compared to 6, 6, and 7 for the negative culture results of the same techniques. The sum of the discordant frequencies between ZN and FM, and between ZN and PiLED were higher relative to those between ZN and CY.

Table 3: Matched sample frequency cells for the agreement and discordance between the standard of care (ZN smear) and FM, CY and PiLED smears on the culture positive and negative specimens

| | | | FM | | CY | | PiLED | |
|---|---|---|---|---|---|---|---|---|
| Culture Positive | | | P | N | P | N | P | N |
| A: n= 72 | ZN | P | 35 | 1 | 34 | 2 | 35 | 1 |
| | | N | 9 | 27 | 05 | 31 | 08 | 28 |
| B: n= 81 | ZN | P | 29 | 0 | 29 | 0 | 29 | 0 |
| | | N | 7 | 45 | 4 | 48 | 10 | 42 |
| C: n= 68 | ZN | P | 29 | 2 | 31 | 0 | 29 | 2 |
| | | N | 6 | 31 | 2 | 35 | 10 | 27 |
| Culture Negative | | | | | | | | |
| A: n= 78 | ZN | P | 6 | 2 | 7 | 2 | 9 | 0 |
| | | N | 7 | 63 | 4 | 65 | 12 | 57 |
| B: n= 69 | ZN | P | 11 | 1 | 6 | 2 | 14 | 0 |
| | | N | 10 | 47 | 4 | 57 | 10 | 45 |
| C: n= 82 | ZN | P | 11 | 1 | 10 | 1 | 13 | 0 |
| | | N | 12 | 58 | 6 | 65 | 10 | 59 |

P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor florescence microscopy; CY=CyScope florescence microscopy and PiLED= Primo Star iLED florescence microscopy
A: cultured samples decontaminated for 10 minutes; B: cultured samples decontaminated for 15 minutes; C: cultured samples decontaminated for 20 minutes

The sensitivity of the ZN technique was consistently less compared to any of the florescence techniques measured against all the three gold standards. The florescence method FM was: 11.1%, 8.6% and 5.9; CY was: 4.2%, 4.9% and 3.1% while PiLED was 9.7%, 12.3%, and 11.8% more sensitive than ZN microscopy ( $P_{fm}$= 0.027, 0.023, 0.289; $P_{cy}$ = 0.450, 0.134, 0.479 and $P_{iled}$ =0.046, 0.004, 0.043). Conversely, the FM microscopy was: 10.2%, 13.1% and 14.6%; CY was: 2.5% 2.5% and 3.6%; PiLED was: 16.6%, 1.4% and 14.6% less specific compared to the ZN microscopy ( $P_{fm}$= 0.182, 0.016, 0.006; $P_{cy}$ = 0.684, 0.684, 0.131 and $P_{iled}$ =0.006, 0.131, 0.004) and also less predictive compared to the ZN (Table 4)

Table 4. Summary measures of performance of the direct smear diagnostic tests for 150 matched samples and the statistical difference in sensitivity and specificity of FM, CY and PiLED compared to ZN

| Gold Stand | Direct Smear | Sn | Sp | PPV | NPV | SnD [95% CI] | p-value* | SpD [95% CI] | p-value* |
|---|---|---|---|---|---|---|---|---|---|
| A | ZN | 50.0 | 89.7 | 81.8 | 66.0 | | | | |
| | FM | 61.1 | 79.5 | 73.3 | 68.9 | 11.1 [3.0, 19.0] | 0.027 | 10.2 [2.5, 17.4] | 0.182 |
| | CY | 54.2 | 87.2 | 79.6 | 67.3 | 4.2 [-2.9, 11.3] | 0.450 | 2.5 [-3.6, 8.6] | 0.684 |
| | PiLED | 59.7 | 73.1 | 67.2 | 66.3 | 9.7 [1.9, 17.5] | 0.046 | 16.6 [8.6, 24.6] | 0.001 |
| B | ZN | 35.8 | 78.3 | 65.9 | 50.9 | | | | |
| | FM | 44.4 | 65.2 | 60.0 | 50.0 | 8.6 [2.4, 14.8] | 0.023 | 13.1 [3.9, 22.1] | 0.016 |
| | CY | 40.7 | 76.8 | 67.3 | 52.5 | 4.9 [0.2, 9.6] | 0.134 | 2.5 [-4.4, 9.4] | 0.684 |
| | PiLED | 48.1 | 63.8 | 60.9 | 51.2 | 12.3 [4.9, 19.6] | 0.004 | 1.4 [-5.5, 7.9] | 0.683 |
| C | ZN | 45.6 | 84.1 | 70.5 | 65.1 | | | | |
| | FM | 51.5 | 69.5 | 58.3 | 63.3 | 5.9 [-2.1, 13.9] | 0.289 | 14.6 [6.6, 22.6] | 0.006 |
| | CY | 48.5 | 80.5 | 67.3 | 65.3 | 3.1 [-0.9, 7.1] | 0.479 | 3.6 [-2.6, 9.8] | 0.131 |
| | PiLED | 57.4 | 69.5 | 60.9 | 66.3 | 11.8 [2.2, 21.4] | 0.043 | 14.6 [7.5, 21.7] | 0.004 |

Sn: Sensitivity; Sp: Specificity; PPV: positive predictive value; NPV; Negative predictive value
SnD: Sensitivity difference; percentage points above ZN performance
SpD: Specificity difference; percentage points below ZN performance
CI: Confidence Interval; *: Chi-square p-value generated from McNemar's test

**Discussion**

The findings of this study further confirm the previously reported superior performance of florescence microscopy over the conventional ZN technique for AFB detection (18-20). The direct smear staining sensitivities of FM and PiLED florescence techniques are on the average 10% more sensitive than the standard of care (ZN method). The reverse is however true for specificity, the florescence methods are less specific than ZN by similar percentage points and these differences are statistically significant. Similar trends of high sensitivity and low specificity were observed in the performance of florescence microscopy in comparison to the conventional Ziehl Neelsen technique (20-22) Within the florescence techniques FM and PiLED have similar performance measures with little or no differences which is in agreement with the reported findings by Marais et al.(23) The CY technique appears to be less sensitive compared to FM and PiLED but on the average have 4% staining sensitivity advantage over the conventional ZN. Similar findings were reported from a resource constrained setting with comparable optimal technical conditions in which such differences were found to be marginally significant statistically. (16). However, both CY and ZN staining techniques have higher specificities compared to FM and PiLED across all the three reference (gold) standards used.

Although less sensitive, the ZN staining seems to be the most predictive: a positive test has the highest chance of being a true case of TB while a negative test is less likely to harbor the disease. The CY has similar predictive probabilities; however, these measures are affected by disease burden. In high prevalence settings, the positive predictive value of a test increases because it is more likely that those who test positive truly have disease than if the test was performed in settings with low prevalence. Likewise, in settings with low prevalence it is more likely that those who test negative truly do not have the disease. In contrast, sensitivity and specificity measures are not influenced by disease prevalence. The search for an optimal tuberculosis diagnostic test is motivated by the unacceptably low sensitivity of the conventional ZN smear microscopy over which florescence microscopy has shown consistent superiority. In this setting, the TB prevalence is high and may be driven by HIV. Priority should therefore be given to sensitivity over predictive value since failure to detect TB in patients co-infected with HIV is more likely to increase morbidity and mortality among the HIV infected persons than false detection of TB.

In addition to improved TB case detection in samples with low-density bacilli, which is aided by its low-power objective, previous studies reported that florescence microscopy requires less than 25% of the time taken to read slides using the conventional ZN technique-meaning a microscopist can examine 4 times the number of slides per day with florescence technique.(16, 24) However, the use of mercury vapor or halogen lamp as the source of light in the standard florescence microscopy technique (FM) is expensive and requires frequent replacement because of their shorter life span making it economically inefficient for use in resource constrained settings. The LED based microscopes (CY and PiLED) on the other hand are structurally built to overcome the hard operational environment of RLS where continuous supply of electricity, sufficient equipment and trained personnel are a frequent challenge. The LED microscopes use lamps that are inexpensive with much longer life span. They are also simple to operate; and no dark room required.

The scanty-positive findings are more commonly seen with ZN technique and least with the FM. This could be attributed to the low-power objective of the florescence techniques that allow the field to be seen larger than in the conventional ZN light microscopy. Some of the samples studied may have come from HIV co-infected patients with low-density bacilli yielding scanty AFBs on the conventional ZN microscopy earning a grade of 1+ or more when viewed under the florescence techniques. However, the florescence techniques recorded the highest numbers of false positives compared to the ZN judging from the reported frequencies in the decision matrix table. This is expected because the low-threshold of the florescence techniques derived from their low-power objective could allow naturally florescence particles present in the sputum, certain spores, fungi to appear AFB positive which may otherwise be negative on ZN microscopy.

As expected, the digestion and decontamination with n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) produces the highest yield of AFB in the sputum exposed to the reagent for 15 minutes. This shows that the manufacturer's recommended exposure time of 15 minutes works well in the hard operational environment of a resource limited setting. The 10 minutes alternative yielded a little less AFB than the 15 minutes duration probably because the time is not enough to allow complete digestion of the thick sputum which allows the release of mycobacteria and their subsequent concentration by centrifugation. The 20 minutes exposure on the other hand might have been prolonged beyond the optimal duration necessary and the toxic effect of the reagent kills not only the contaminating normal flora that may overgrow in cultures making it hard to detect the presence of the mycobacteria but also the mycobacteria itself. The success of this process is dependent on the concentration of the reagent, the exposure time, centrifugation and the temperature selected. In this case all other factors were kept constant and only the exposure time was altered.

The design of this study allows test performance assessment under routine (real) resource constrain settings. The point estimates obtained are more likely to represent the expected outcomes in similar settings with high prevalence of TB. Blinding the readers on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of the direct smear results. However, our study had a few limitations: We did not obtain participants' HIV status and therefore unable to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures. The time taken to read slides was also not reflected in the analysis because only estimates of the time taken to read the slides were recorded since the different technicians that read the slides had different proficiency levels and some accurately read the slides much faster than others.

In summary, the high sensitivity for AFB detection shown by the inexpensive light-emitting diode based Primo Star iLED florescence microscope in the hard operational conditions of a resource limited setting underscores the potentials for its scalability to remote clinics in similar settings.. This will increase TB detection and improve TB treatment uptakes in high burden settings with scarce resources. The optimal exposure duration for decontamination of sputum samples prior to examination and culture using the n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) reagent in this setting is 15 minutes.

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## References

1. De Cock KM, Simone PM, Davison V, Slutsker L. The new global health. Emerg Infect Dis. 2013 Aug;19(8):1192-7.

2. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Review]. 2003 May 12;163(9):1009-21.

3. Young DB, Perkins MD, Duncan K, Barry CE, 3rd. Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest. [Research Support, Non-U.S. Gov't Review]. 2008 Apr;118(4):1255-65.

4. Getahun H, Harrington M, O'Brien R, Nunn P. Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. Lancet. [Review]. 2007 Jun 16;369(9578):2042-9.

5. Somoskovi A, Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M. Lessons from a proficiency testing event for acid-fast microscopy. Chest. [Research Support, U.S. Gov't, P.H.S.]. 2001 Jul;120(1):250-7.

6. Van Deun A, Salim AH, Cooreman E, Hossain MA, Rema A, Chambugonj N, et al. Optimal tuberculosis case detection by direct sputum smear microscopy: how much better is more? Int J Tuberc Lung Dis. [Evaluation Studies]. 2002 Mar;6(3):222-30.

7. Cole EC, Cook CE. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. Am J Infect Control. [Review]. 1998 Aug;26(4):453-64.

8. Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin Microbiol Rev. [Review]. 2011 Apr;24(2):314-50.

9. Monkongdee P, McCarthy KD, Cain KP, Tasaneeyapan T, Nguyen HD, Nguyen TN, et al. Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. Am J Respir Crit Care Med. [Evaluation Studies Research Support, U.S. Gov't, Non-P.H.S.]. 2009 Nov 1;180(9):903-8.

10. Cheng S, Chen W, Yang Y, Chu P, Liu X, Zhao M, et al. Effect of Diagnostic and Treatment Delay on the Risk of Tuberculosis Transmission in Shenzhen, China: An Observational Cohort Study, 1993-2010. PLoS One. 2013;8(6):e67516.

11. Tadesse T, Demissie M, Berhane Y, Kebede Y, Abebe M. Incidence of smear-positive tuberculosis in Dabat, northern Ethiopia. Int J Tuberc Lung Dis. [Research Support, U.S. Gov't, Non-P.H.S.]. 2013 May;17(5):630-5.

12. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. [Comparative Study Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Review]. 2006 Sep;6(9):570-81.

13. Hooja S, Pal N, Malhotra B, Goyal S, Kumar V, Vyas L. Comparison of Ziehl Neelsen & Auramine O staining methods on direct and concentrated smears in clinical specimens. Indian J Tuberc. [Comparative Study]. 2011 Apr;58(2):72-6.

14. Prasanthi K, Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. Indian J Med Microbiol. [Comparative Study]. 2005 Jul;23(3):179-81.

15. Selvakumar N, Rahman F, Rajasekaran S, Narayanan PR, Frieden TR. Inefficiency of 0.3% carbol fuchsin in ziehl-neelsen staining for detecting acid-fast bacilli. J Clin Microbiol. [Comparative Study Evaluation Studies Research Support, Non-U.S. Gov't]. 2002 Aug;40(8):3041-3.

16. Lehman LG, Ngapmen Yamadji AL, Ngo Sack F, Bilong Bilong CF. The CyScope(R) fluorescence microscope, a reliable tool for tuberculosis diagnosis in resource-limited settings. Am J Trop Med Hyg. [Research Support, Non-U.S. Gov't]. 2010 Oct;83(4):906-8.

17. Hawass NE. Comparing the sensitivities and specificities of two diagnostic procedures performed on the same group of patients. Br J Radiol. [Comparative Study]. 1997 Apr;70(832):360-6.

18. Hung NV, Sy DN, Anthony RM, Cobelens FG, van Soolingen D. Fluorescence microscopy for tuberculosis diagnosis. Lancet Infect Dis. [Comment Letter]. 2007 Apr;7(4):238-9; author reply 9-40.

19. Van Deun A, Chonde TM, Gumusboga M, Rienthong S. Performance and acceptability of the FluoLED Easy module for tuberculosis fluorescence microscopy. Int J Tuberc Lung Dis. [Comparative Study Evaluation Studies Research Support, U.S. Gov't, Non-P.H.S.]. 2008 Sep;12(9):1009-14.

20. Ulukanligil M, Aslan G, Tasci S. A comparative study on the different staining methods and number of specimens for the detection of acid fast bacilli. Mem Inst Oswaldo Cruz. [Comparative Study]. 2000 Nov-Dec;95(6):855-8.

21. Cuevas LE, Al-Sonboli N, Lawson L, Yassin MA, Arbide I, Al-Aghbari N, et al. LED fluorescence microscopy for the diagnosis of pulmonary tuberculosis: a multi-country cross-sectional evaluation. PLoS Med. [Controlled Clinical Trial Multicenter Study Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S.]. 2011 Jul;8(7):e1001057.

22. Habtamu M, van den Boogaard J, Ndaro A, Buretta R, Irongo CF, Lega DA, et al. Light-emitting diode with various sputum smear preparation techniques to diagnose tuberculosis. Int J Tuberc Lung Dis. [Comparative Study Research Support, Non-U.S. Gov't]. 2012;16(3):402-7.

23. Marais BJ, Brittle W, Painczyk K, Hesseling AC, Beyers N, Wasserman E, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clin Infect Dis. [Comparative Study Evaluation Studies]. 2008 Jul 15;47(2):203-7.

24. Ba F, Rieder HL. A comparison of fluorescence microscopy with the Ziehl-Neelsen technique in the examination of sputum for acid-fast bacilli. Int J Tuberc Lung Dis. [Comparative Study]. 1999 Dec;3(12):1101-5.

**Checklist for optimizing tuberculosis detection manuscript submitted to BMJ open**

| Section and Topic | Item # | | On page # |
|---|---|---|---|
| TITLE/ABSTRACT/ KEYWORDS | 1 | *Optimizing Mycobacterium tuberculosis detection in resource limited settings*: a comparison between different types of florescence smear microscopy techniques with the traditional Zeal Nielsen's (ZN) smear microscopy under routine working conditions of a resource limited setting. | 1 |
| INTRODUCTION | 2 | The diagnostic accuracies of the standard but more expensive mercury vapor lamp (FM) florescence microscope; the newer, less expensive LED based CyScope® (CY); and the Primo Star iLED (PiLED) florescence microscopes were compared to that of the conventional light ( ZN) microscope against the gold (reference) standard of liquid culture in automated BACTEC MGIT 960™ machine | 2 |
| METHODS | | | |
| *Participants* | 3 | 450 sputum samples from 150 new patients with clinical diagnosis of pulmonary tuberculosis were studied from the main tuberculosis case referral center in northern Nigeria. | 3 |
| | 4 | Participant recruitment: sputum samples from new patients with clinical diagnosis of pulmonary tuberculosis based on presenting symptoms independent of the outcome of the routine (standard of care TB test) were selected for this study. | 3 |
| | 5 | Participant sampling: Sputum samples of consecutive new but de-identified patients with clinical diagnosis of pulmonary tuberculosis were sampled. | 3 |
| | 6 | Data collection: This was a cross-sectional study where three sputum samples collected from patients over 24 hour period (spot-morning-spot) for the purpose of routine clinical care were used. | 3 |
| *Test methods* | 7 | The reference standard was sputum culture in Mycobacterium Growth Indicator Tubes (MGIT) and incubated in the automated BACTEC MGIT 960™ machine. Liquid cultures are more sensitive, fast and reliable techniques for the diagnosis of pulmonary tuberculosis | 4 |
| | 8 | The three sputum samples collected from each patient were pooled into a single specimen. Four direct smears were made from each of the pooled specimen. The first slide was stained with Ziehl Neelsen (ZN) while the second, third and fourth slides were Auramine-rhodomine stained stain and examined under FM, CY and PiLED florescence microscopes respectively. | 3 |

| | | | |
|---|---|---|---|
| | 9 | Positive smears were graded as scanty if there were: 1-9 AFBs in at least 100 fields; +1: 10-99 in 100 fields; +2: 1-10 per field in at least 50 fields; and +3: if there were more than 10 per field in at least 20 fields. Absence of any AFB was graded as negative smear.<br>Cultures that indicated positive growth were removed from the MGIT machine and the presence of AFB confirmed by ZN smear microscopy. Those that do not indicate growth after 42 days were classified as negatives as recommended by the manufacturer and as was the standard practice. | 3-4 |
| | 10 | Three experts were involved with the execution and interpretation of the smear microscopy tests while two were responsible for the reference (culture) tests. All experts hold a minimum of bachelor's degree in microbiology and had worked for at least 2 years prior to the study conduct. | 4 |
| | 11 | Experts were blinded on the clinical diagnoses and outcome of the tests done for clinical care purposes. | 4 |
| *Statistical methods* | 12 | Diagnostic accuracies were measured using decision matrix tables and matched sample tables for positive and negative final outcomes as previously recommended for comparative analysis of diagnostic procedures performed on same samples (patients) by Hawass NE in Br J Radiol. 1997 Apr;70(832):360-6. Differences in tests performances together with their 95% confidence intervals were evaluated using the McNemar's test. | 4-5 |
| | 13 | Routine laboratory quality assurance measures were used to assess tests reliability, not reported in this manuscript. | 0 |
| RESULTS | | | |
| *Participants* | 14 | This study was conducted from July 3, to September 26, of 2009 | 3 |
| | 15 | Clinical and demographic information of participants was excluded to minimize bias and ensure complete de-identification of data | 3 |
| | 16 | Main inclusion criteria were clinical diagnoses of TB. Culture results of 19 patients were contaminated and therefore excluded from the analyses. Samples from 90 patients with clinical diagnoses of TB were incomplete and were similarly excluded. | 3 |
| *Test results* | 17 | There was no TB treatment administered within the 24 hour interval in which samples were collected. The index and reference tests were performed on the same sample. | 3 |
| | 18 | Disease severity was not graded in this study. However, there were patients with clinical diagnoses of pulmonary diseases other than tuberculosis. Most of those patients were not requested to provide sputum samples for tuberculosis test and such samples were not targets of this study. | 0 |

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|---|---|---|---|
| | 19 | A cross tabulation of the results of the index tests by the results of the reference standard were provided in Tables 1 and 2. | 7-8 |
| | 20 | The tests were performed on sputum samples provided by patients, no intervention was involved and therefore no adverse events monitored. | 0 |
| *Estimates* | 21 | Details of the estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals) were provided in Table 4 of the manuscript. | 10 |
| | 22 | The outcomes of the index and reference standard test were binary (yes, or no). There were no outliers, no missing data. | 0 |
| | 23 | Variability of diagnostic accuracy between the smear microscopy readers was not evaluated, a smear was positive if AFBs were seen by all the three readers. | 4 |
| | 24 | Estimates of test reproducibility was not evaluated | 0 |
| DISCUSSION | 25 | The high sensitivity for AFB detection shown by the inexpensive light-emitting diode based Primo Star iLED florescence microscope in the hard operational conditions of a resource limited setting underscores the potentials for its scalability to remote clinics with similar operational conditions. This will increase TB detection and improve TB treatment uptakes in high burden settings with scarce resources. The optimal exposure duration for decontamination of sputum samples prior to examination and culture using the n-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) reagent in this setting is 15 minutes. | 13 |

# Optimizing Mycobacterium tuberculosis detection in resource limited settings

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# Optimizing Mycobacterium tuberculosis detection in resource limited settings

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## Abstract

**Objectives**: The light-emitting diode (LED) fluorescence microscopy has made acid-fast bacilli (AFB) detection faster and efficient although its optimal performance in resource limited settings is still being studied. We assessed the optimal performances of light and fluorescence microscopy in routine conditions of a resource limited setting and evaluated the digestion time for sputum samples for maximum yield of positive cultures.

**Design**: Cross-sectional study

Setting: Facility-based involving samples of routine patients receiving tuberculosis treatment and care from the main tuberculosis case referral center in northern Nigeria.

Participants: The study included 450 sputum samples from 150 new patients with clinical diagnosis of pulmonary tuberculosis.

**Methods**: The 450 samples were pooled into 150 specimens, examined independently with mercury vapor lamp (FM), LED CysCope® (CY) and Primo Star iLED (PiLED) fluorescence microscopies, and with the Ziehl Neelsen (ZN) microscopy to assess the performance of each technique compared to liquid culture. The cultured specimens were decontaminated with BD Mycoprep (4%NaOH-1%NLAC and 2.9% sodium citrate) for 10, 15 and 20 minutes before incubation in Mycobacterium Growth Incubator Tube (MGIT) system and growth examined for acid-fast bacilli (AFB)

**Results**: Of the 150 specimens examined by direct microscopy: 44 (29%), 60 (40%), 49 (33%) and 64 (43%) were AFB positive by ZN, FM, CY, and iLED microscopy respectively. Digestion of sputum samples for 10, 15 and 20 minutes yielded mycobacterial growth in 72 (48%), 81 (54%) and 68 (45%) of the digested samples respectively after incubation in the MGIT system.

**Conclusions**: In routine laboratory conditions of a resource-limited setting, our study has demonstrated the superiority of fluorescence microscopy over the conventional ZN technique. Digestion of sputum samples for 15minutes yielded more positive cultures


**Keywords**: Tuberculosis, Microbiology, Respiratory infections

**Word count**: 3,399

**Article focus**:

1. What is the optimal performance of the new light-emitting diode (LED) florescence microscopy in routine conditions of a resource limited setting and how does it compare to the traditional light microscopy using Ziehl Neelsen (ZN) technique for AFB smear microscopy?
2. What is the optimal digestion time for sputum samples for maximum yield of positive cultures in routine conditions of a resource limited setting?
3. Despite the operational challenges of resource limited settings florescence microscopy has better optimal performance compared to the traditional light microscopy using Ziehl Neelsen (ZN) technique for AFB smear microscopy.

**Significance**:

1. Most of the settings with highest burden of Tuberculosis in the world are limited in resource needed to effectively detect and manage cases of the disease.
2. The WHO has recommended the phasing out of the simple, inexpensive but less sensitive ZN technique with the more sensitive florescence technique for better TB detection in all settings.
3. Evaluating the performance of the more sensitive florescence microscopy against the less sensitive ZN microscopy in unaltered working conditions of resource limited settings is relevant for effective TB control.

**Strength and Limitations**:

1. The design of this study allows test performance assessment under routine (real) resource constrain settings.
2. Blinding the microscopist on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of direct smear results
3. However, our inability to obtain participant HIV status deprived us the ability to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures.

## Introduction

Globally, an estimated 1.4 million death occurred in 2011 as a result of infection with tuberculosis, one-fourth of the death was associated with HIV infection and most of it in resource limited settings (1) where the burden of HIV infection is high. In Africa, TB is the leading cause of death among HIV infected persons as the continent harbors eighty percent of the world HIV-TB cases (2). Nigeria and the Republic of South Africa are among the top five TB burden nations of the world. Sensitive, specific, and inexpensive point-of-care diagnostic tools for rapid TB detection are lacking posing challenges to the optimal diagnosis of TB in resource limited settings (RLS), particularly among persons with HIV infections whose sputum specimen often lack sufficient quantity of acid-fast bacilli (AFB) detectable on routine smear examination. (3, 4)

The simplicity, inexpensiveness and predictive power of the Ziehl Neelsen sputum smear microscopy make it the applicable laboratory diagnostic tool of choice for tuberculosis in resource limited settings. (5) A properly done positive sputum-smear is highly predictive of active TB (6, 7). Unfortunately, the sensitivity of sputum-smear microscopy is low and its performance is often affected by lack of proper maintenance of equipment; trained manpower; and quality assurance system in settings where resources are scarce. (8) Even in centers with good quality assurance measures, smear microscopy detects only 40-50% of TB cases compared to about 80% yield by culture (9). However, it remains the only available tool for bacteriologic diagnosis of TB in most RLS. Culture techniques are highly sensitive and specific, but the cost, technical complexity and time delay before results are available make culture not scalable for rapid detection and treatment of tuberculosis. Recent advances have made it possible to use molecular technology to detect Mycobacterial deoxyribonucleic acid (DNA) with great accuracy. However these technologies still require a fairly developed laboratory with a reliable source of electricity.

Detection of smear positive cases is the priority in TB control programs. These cases are infectious and contribute substantially to the transmission of the disease. (10, 11) In recent years, several new laboratory techniques have been developed to significantly improve the ability to diagnose active TB in populations. Fluorescence microscopy improves the sensitivity of sputum-smear detection of TB and has good specificity relative to the conventional ZN method. Inexpensive light-emitting diode-based (LED) fluorescence

microscopes have been developed to make microscopy of smears stained by Auramine fluorescence dye easier, cheaper and faster with potentials for scalability to remote clinics in RLS. (5, 12-14).

This study compared the performance of the standard but more expensive mercury vapor lamp (FM) fluorescence microscope; the newer, less expensive LED based CysCope® (CY) (Partec, Görlitz, Germany); and the Primo Star iLED (PiLED) (Carl Zeiss Inc. Germany) fluorescence microscopes in comparison to the conventional light ( ZN) microscope against the gold (reference) standard of liquid culture in automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems) for the detection of AFB from sputum samples of patients with clinical pulmonary tuberculosis (TB). In addition, we evaluated the optimal digestion time with *Mycoprep (4%NaOH-1%NLAC and 2.9% sodium citrate)* for liquid culture in this population. Prolonged decontamination time is reported to reduce the number of viable bacilli and different digestion methods yield variable number of positive cultures (15-17).

## Methods

*Study population*

Three sputum samples (spot-morning-spot) were collected from 150 TB treatment naïve, consecutive patients with clinical diagnosis of pulmonary tuberculosis at the National TB and Leprosy Training Center (NTBLTC), Zaria, Nigeria. Routine and referred cases of TB from northern Nigerian region receive TB treatment and care at this facility. The spot-morning-spot sample collection was the standard routine for the diagnosis of TB in Nigeria at the time of the study. The flow chart in **Figure 1** provides an overview of the study design. Ethical review was waived because in the opinion of the study center review committee there was no potential risk to participants' safety, privacy or confidentiality since there was no formal contact between investigators and participants either directly (interview, questionnaires, etc.) or indirectly (medical records, personal identifiers etc.). The sputum specimens provided for routine clinical care services were completely anonymized before they were analyzed for the study and there was no risk that the pooled samples can be de-anonymized through data linkages. This study was conducted from July to September of 2009.

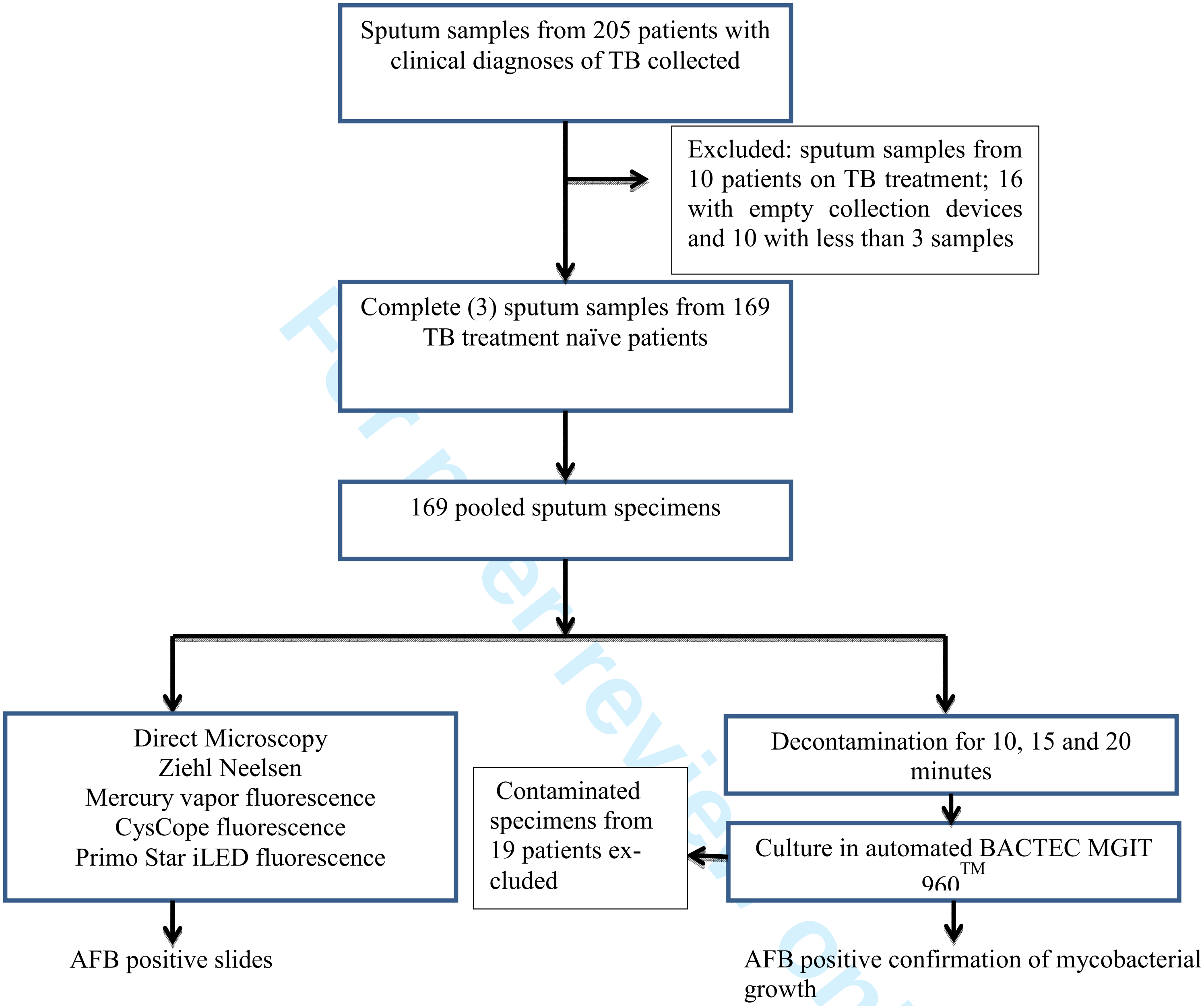


**Figure 1**. Flow chart for the study design involving 450 sputum samples from 150 patients with clinical TB

*Smear Microscopy*

The three sputum samples from each patient were pooled. Four direct smears were made from each of the pooled sputum samples with applicator sticks on clean grease-free slides; measuring 2 cm x 3 cm, and not too thick. The first slide was stained with Ziehl Neelsen (ZN) stain according to the World Health Organization (WHO) recommended protocol described elsewhere. (18) Briefly, the smear was air dried and fixed by gently passing it over a flame 2-3 times. One percent strong carbol-fuchsin was applied to the slide and heated with a Bunsen flame intermittently 3 times and allowed to stain for 15 minutes. The stain was rinsed off with tap water and 3% acid alcohol was applied on the smear for 3 minutes. Methylene blue (0.3%) was then added for up to 1 minute and rinsed off with tap water. Positive and negative controls were included in the process. Slide examination was done with light microscope at 1,000x magnification and the AFBs identified were graded according to the International Union against Tuberculosis and Lung Disease (IUATLD) and the WHO smear grading scale. (19)

The second, third and fourth slides were air-dried, heat-fixed and stained with Auramine O-phenol for 15 minutes rinsed with tap water, decolorized with 1 percent acid alcohol for 2 minutes then rinsed with water and counterstained with 0.1 percent potassium permanganate for 2 minutes. The slides were rinsed with water then allowed to dry in air before examination under FM, CY and PiLED microscopes respectively. The AFBs were visualized and graded at magnifications of 200× and 400× according to a protocol describe by Lehman (20). A total of 100 microscopic fields were examined before a slide is recorded as positive (if at least an AFB was seen) or negative (if no AFB was seen). Positive smears were graded as scanty (actual) if there were: 1-9 AFBs in at least 100 fields; +1: 10-99 in 100 fields; +2: 1-10 per field in at least 50 fields; and +3: if there were more than 10 per field in at least 20 fields. Absence of any AFB was graded as negative smear. Each slide was examined by three independent readers to ascertain the presence of AFB and grade positive smears. The slide readers were blinded on the clinical and laboratory diagnoses of the subjects whose samples were studied.

*Specimen culture*

Each of the 150 pooled sputum specimens were decontaminated with BD Mycoprep$^{TM}$ (Beckton Dickinson Diagnostic Systems, Sparks, Maryland, USA) which consists of 1% N-acetyl-L-cysteine (NALC), 4% sodium hydroxide and 2.9% sodium citrate. An equal amount of Mycoprep was added to the sputum, homogenized and

allowed to act for 10, 15 and 20 minutes respectively. After the respective digestion period, phosphate buffered saline was added to stop the digestion and decontamination reaction. This was then centrifuged under refrigerated conditions with uniform centrifugation time of 15 minutes and a Relative Centrifugal force (RCF) of 3000 x g. The supernatants were discarded and the sediments re-suspended with 1 ml of phosphate buffered saline to make a final volume of 2 ml. Then 0.5 ml of each was inoculated into Mycobacterium Growth Indicator Tubes (MGIT) and incubated in the automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems), which monitors growth. Positive and negative control culture tubes were also set up along each test using *Mycobacterium tuberculosis* H37Ra (ATCC 25177 – attenuated strain) and sterile distilled water respectively.

The cultures that indicated positive growth were removed from the machine and the presence of AFB confirmed by ZN smear microscopy. Those that do not indicate growth after 42 days were classified as negatives as recommended by the manufacturer and as was the standard practice. All cultures were done at Zankli Research laboratory while all microscopy were performed at the NTBLTC, Zaria to minimize interference with the outcome of culture or direct microscopy results.

*Statistical analysis*

The raw data generated from the smear microscopic examinations of the 150 matched samples by the four different microscopes as well as the data generated after same samples were cultured following decontaminations (digestions) at 10, 15 and 20 minutes were organized into cell counts in which descriptive values were provided along with frequencies and proportions. Measures of accuracy for the different smear examinations: sensitivity, specificity, positive and negative predictive values were evaluated through decision matrix tables while concordance and disagreements between the microscopic examination by each of the three fluorescence microscopes and the conventional light (ZN) microscope based on the final classification of samples by the gold standards (outcomes MGIT cultures) were examined in matched sample tables for positive and negative final outcomes as previously recommended for comparative analysis of diagnostic procedures performed on same samples (patients) (21). Comparison between the fluorescence microscopies to the conventional ZN microscopy for differences in performance together with their 95% confidence intervals was done using the McNemar's test. Significance of association was set at 0.05 alpha and all probabilities were two tailed.

**Results**

Of the 150 matched (paired) samples, AFBs were detected in: 44 (29%) of the ZN smears examined under light microscope; 60 (40%) of the AR smears examined with FM; 49 (33%) of the AR smears examined with CY; and 64 (44%) AR smears examined with PiLED fluorescence microscopes. MGIT cultures of the 150 specimen yielded 72 (48%), 81 (54%) and 68 (45%) AFB confirmed growths after decontamination for 10 (A), 15 (B) and 20 (C) minutes respectively prior to incubation.).

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Among the positive AFB tests graded, ZN light microscopy produced the highest proportion of samples with scanty AFBs on examination 21/44 (48%), followed by PiLED fluorescence microscopy 12/64 (19%), then CY 8/49 (16%) and FM had the least scanty AFB positive counts 3/60 (5%). However, after the samples with scanty positive AFBs where incubated in A, B and C MGIT culture groups, the proportions of confirmed myco-bacterial growths in the sequence above were as follows: (i) ZN: 14/21 (67%), 9/21 (42%) and 11/21 (52%); (ii) PiLED: 5/12 (42%), 7/12 (58%) and 5/12 (42%); (iii) CY: 6/8 (75%), 4/8 (50%) and 3/8 (38%); lastly (iv) FM: 0/3 (0%), 1/3 (33%) and 1/3 (33%). A summary of the positive AFB outcomes from the direct smear examina-tions by microcopy types, grades and the proportions of graded smears confirmed by the MGIT cultures were provided in **Table 1**.

**Table 1**. Proportions of positive graded direct smears confirmed by cultured specimen decontaminated at 10 (A), 15 (B) and 20(C) minutes obtained from 150 matched samples

| Direct smear (DS) | Grade | Frequency | MGIT cultured specimen (Gold standards) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A | | B | | C | |
| | | | n | % | n | % | n | % |
| ZN | scanty | 21 | 14 | 66.7 | 9 | 42.1 | 11 | 52.4 |
| | +1 | 13 | 12 | 92.3 | 11 | 84.6 | 10 | 76.9 |
| | +2 | 5 | 5 | 100.0 | 4 | 80.0 | 5 | 100.0 |
| | +3 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| FM | Scanty | 3 | 0 | 0.0 | 1 | 33.3 | 1 | 33.3 |
| | +1 | 34 | 24 | 70.6 | 19 | 55.9 | 17 | 50.0 |
| | +2 | 18 | 16 | 88.9 | 12 | 66.7 | 13 | 72.2 |
| | +3 | 5 | 4 | 80.0 | 4 | 80.0 | 4 | 80.0 |
| CY | Scanty | 8 | 6 | 75.0 | 4 | 50.0 | 3 | 37.5 |
| | +1 | 30 | 22 | 73.3 | 18 | 60.0 | 19 | 63.3 |
| | +2 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| | +3 | 6 | 6 | 100.0 | 6 | 100.0 | 6 | 100.0 |
| PiLED | Scanty | 12 | 5 | 41.7 | 7 | 58.3 | 5 | 41.7 |
| | +1 | 36 | 22 | 61.1 | 20 | 55.6 | 18 | 50.0 |
| | +2 | 13 | 13 | 100.0 | 10 | 76.9 | 13 | 100.0 |
| | +3 | 3 | 3 | 100.0 | 2 | 66.7 | 3 | 100.0 |

ZN: Ziehl Neelsen microscopy using the conventional light microscope
FM: Fluorescence microscopy with mercury vapor lamp microscope
CY: Fluorescence microscopy with CysCope light emitting diode microscope
PiLED: Fluorescence microscopy with Primo Star iLED light emitting diode microscope

A comparison of the direct smear tests results with the final culture outcomes to measure the performances of the direct smear techniques against the gold standards showed that of the 44 AFB positive samples identified by the ZN microscopy; 36 (81.8%), 29 (65.9%) and 31 (70.5%) were culture confirmed after 10, 15 and 20 minutes decontamination respectively. Similarly, of the 60, 49 and 64 AFB positive samples identified by the FM, CY and PiLED microscopy techniques the frequencies and proportions of the culture confirmed samples were: 44 (73.3%) 36 (60.0%) and 35 (58.3%) for FM; 39 (79.6%), 33 (67.3%) and 33 (67.3%) for CY;

and 43 (67.2%) 39 (56.3%) and 39 (56.3%) for PiLED at decontamination times of 10, 15 and 20 minutes respectively. There were near identical frequency distributions for the true positive (TP), false positive (FP), false negative (FN) and true negative (TN) for the ZN and CY microscopy techniques while the FM microscopy yielded a pattern of distribution similar to that of PiLED technique as shown in the decision matrix of **Table 2**.

**Table 2**. Decision matrix for the evaluation of performance of the direct smear methods against the different gold standards for the 150 matched samples

| Direct Smear (DS) | DS Outcome | Gold standards: MGIT cultures | | | | | |
|---|---|---|---|---|---|---|---|
| | | A : culture positive =72 | | B: culture positive =81 | | C: culture positive = 68 | |
| | | P | N | P | N | P | N |
| ZN | P | 36 | 08 | 29 | 15 | 31 | 13 |
| DS+ = 44 | N | 36 | 70 | 52 | 54 | 37 | 69 |
| FM | P | 44 | 16 | 36 | 24 | 35 | 25 |
| DS+ =60 | N | 28 | 62 | 45 | 45 | 33 | 57 |
| CY | P | 39 | 10 | 33 | 16 | 33 | 16 |
| DS+ =49 | N | 33 | 68 | 48 | 53 | 35 | 66 |
| PiLED | P | 43 | 21 | 39 | 25 | 39 | 25 |
| DS+ =64 | N | 29 | 57 | 42 | 44 | 29 | 57 |

Culture specimen decontaminated by NaOH-NALC : A for 10 minutes; B for 15minutes; C for 20 minutes
DS+ = number of samples (out of 150 samples) that were AFB positive by direct smear
P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor fluorescence microscopy; CY=CysCope fluorescence microscopy and PiLED= Primo Star iLED fluorescence microscopy

The levels of agreement and differences between the convention ZN microscopy and each of the fluorescence microscopy techniques with respect to the final culture results were demonstrated by the concordance and discordance cells displayed in **Table 3**. Cells were discordant if the outcomes of the two techniques disagreed otherwise they were concordant. The statistical significance of the difference in the performance of the techniques compared was derived from the discordant cell. The sum of the frequencies of the discordant cells for example that explained the differences between ZN and CY were 7, 4 and 2 for the positive culture results which showed less consistent divergence compared to 6, 6, and 7 for the negative culture results of the same

techniques. The sum of the discordant frequencies between ZN and FM, and between ZN and PiLED were higher relative to those between ZN and CY.

**Table 3**: Matched sample frequency cells for the agreement and discordance between the standard of care (ZN smear) and FM, CY and PiLED smears on the culture positive and negative specimens

| | | | FM | | CY | | PiLED | |
|---|---|---|---|---|---|---|---|---|
| Culture Positive | | | P | N | P | N | P | N |
| A: n= 72 | ZN | P | 35 | 1 | 34 | 2 | 35 | 1 |
| | | N | 9 | 27 | 05 | 31 | 08 | 28 |
| B: n= 81 | ZN | P | 29 | 0 | 29 | 0 | 29 | 0 |
| | | N | 7 | 45 | 4 | 48 | 10 | 42 |
| C: n= 68 | ZN | P | 29 | 2 | 31 | 0 | 29 | 2 |
| | | N | 6 | 31 | 2 | 35 | 10 | 27 |
| Culture Negative | | | | | | | | |
| A: n= 78 | ZN | P | 6 | 2 | 7 | 2 | 9 | 0 |
| | | N | 7 | 63 | 4 | 65 | 12 | 57 |
| B: n= 69 | ZN | P | 11 | 1 | 6 | 2 | 14 | 0 |
| | | N | 10 | 47 | 4 | 57 | 10 | 45 |
| C: n= 82 | ZN | P | 11 | 1 | 10 | 1 | 13 | 0 |
| | | N | 12 | 58 | 6 | 65 | 10 | 59 |

P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor fluorescence microscopy; CY=CysCope fluorescence microscopy and PiLED= Primo Star iLED fluorescence microscopy
A: cultured samples decontaminated for 10 minutes; B: cultured samples decontaminated for 15 minutes; C: cultured samples decontaminated for 20 minutes

The ZN technique was consistently less sensitive compared to any of the fluorescence techniques against any of the gold standards. The FM fluorescence technique was: 11.1%, 8.6% and 5.9%; CY method was: 4.2%, 4.9% and 3.1% while PiLED was 9.7%, 12.3%, and 11.8% more sensitive than ZN microscopy for the different digestion times of 10, 15 and 20 minutes respectively ( $P_{fm}$= 0.027, 0.023, 0.289; $P_{cy}$ = 0.450, 0.134, 0.479 and $P_{iled}$ =0.046, 0.004, 0.043). Conversely, the FM was: 10.2%, 13.1% and 14.6%; CY was: 2.5% 2.5% and 3.6%; and PiLED was: 16.6%, 1.4% and 14.6% less specific compared to the ZN microscopy for the same digestion times above (*Pfm*= 0.182, 0.016, 0.006; $P_{cy}$ = 0.684, 0.684, 0.131 and $P_{iled}$ =0.006, 0.131, and 0.004). They were also less predictive than the ZN technique (**Table 4**).

**Table 4**. Summary measures of performance of the direct smear diagnostic tests for 150 matched samples and the statistical differences in sensitivity and specificity of FM, CY and PiLED in comparison to ZN

| Gold Stand | Direct Smear | Sn | Sp | PPV | NPV | SnD [95% CI] | p-value* | SpD [95% CI] | p-value* |
|---|---|---|---|---|---|---|---|---|---|
| A | ZN | 50.0 | 89.7 | 81.8 | 66.0 | | | | |
| | FM | 61.1 | 79.5 | 73.3 | 68.9 | 11.1 [3.0, 19.0] | 0.027 | 10.2 [2.5, 17.4] | 0.182 |
| | CY | 54.2 | 87.2 | 79.6 | 67.3 | 4.2 [-2.9, 11.3] | 0.450 | 2.5 [-3.6, 8.6] | 0.684 |
| | PiLED | 59.7 | 73.1 | 67.2 | 66.3 | 9.7 [1.9, 17.5] | 0.046 | 16.6 [8.6, 24.6] | 0.001 |
| B | ZN | 35.8 | 78.3 | 65.9 | 50.9 | | | | |
| | FM | 44.4 | 65.2 | 60.0 | 50.0 | 8.6 [2.4, 14.8] | 0.023 | 13.1 [3.9, 22.1] | 0.016 |
| | CY | 40.7 | 76.8 | 67.3 | 52.5 | 4.9 [0.2, 9.6] | 0.134 | 2.5 [-4.4, 9.4] | 0.684 |
| | PiLED | 48.1 | 63.8 | 60.9 | 51.2 | 12.3 [4.9, 19.6] | 0.004 | 1.4 [-5.5, 7.9] | 0.683 |
| C | ZN | 45.6 | 84.1 | 70.5 | 65.1 | | | | |
| | FM | 51.5 | 69.5 | 58.3 | 63.3 | 5.9 [-2.1, 13.9] | 0.289 | 14.6 [6.6, 22.6] | 0.006 |
| | CY | 48.5 | 80.5 | 67.3 | 65.3 | 3.1 [-0.9, 7.1] | 0.479 | 3.6 [-2.6, 9.8] | 0.131 |
| | PiLED | 57.4 | 69.5 | 60.9 | 66.3 | 11.8 [2.2, 21.4] | 0.043 | 14.6 [7.5, 21.7] | 0.004 |

Sn: Sensitivity; Sp: Specificity; PPV: positive predictive value; NPV; Negative predictive value
SnD: Sensitivity difference; percentage points above ZN performance
SpD: Specificity difference; percentage points below ZN performance
CI: Confidence Interval; *: Chi-square p-value generated from McNemar's test

## Discussion

The findings of this study further confirm the previously reported superior performance of fluorescence microscopy over the conventional ZN technique for AFB detection (22-24). The direct smear staining sensitivities of FM and PiLED fluorescence techniques are on the average 10% more sensitive than the standard of care (ZN method). The reverse is however true for specificity, the fluorescence methods are less specific than ZN by similar percentage points and these differences are statistically significant. Similar trends of high sensitivity and low specificity were observed in the performance of fluorescence microscopy in comparison to the conventional Ziehl Neelsen technique (24-26) Within the fluorescence techniques FM and PiLED have similar performance measures with little or no differences which is in agreement with the reported findings by Marais et al.(27) The CY technique appears to be less sensitive compared to FM and PiLED but on the average have 4% staining sensitivity advantage over the conventional ZN. Similar findings were reported from a resource constrained setting with comparable optimal technical conditions in which such differences were found to be marginally significant statistically. (20). However, both CY and ZN staining techniques have higher specificities compared to FM and PiLED across all the three reference (gold) standards used.

Although less sensitive, the ZN staining seems to be the most predictive: a positive test has the highest chance of being a true case of TB while a negative test is less likely to harbor the disease. The CY has similar predictive probabilities; however, these measures are affected by disease burden. In high prevalence settings, the positive predictive value of a test increases because it is more likely that those who test positive truly have disease than if the test was performed in settings with low prevalence. Likewise, in settings with low prevalence it is more likely that those who test negative truly do not have the disease. In contrast, sensitivity and specificity measures are less susceptible to influence by disease prevalence. The search for an optimal tuberculosis diagnostic test is motivated by the unacceptably low sensitivity of the conventional ZN smear microscopy over which fluorescence microscopy has shown consistent superiority. In this setting, the TB prevalence is high and may be driven by HIV. Priority should therefore be given to sensitivity over predictive value since failure to detect TB in patients co-infected with HIV is more likely to increase morbidity and mortality among the HIV infected persons than false detection of TB.

In addition to improved TB case detection in samples with low-density bacilli, which is aided by its low-power objective, previous studies reported that fluorescence microscopy requires less than 25% of the time taken to read slides using the conventional ZN technique-meaning a microscopist can examine 4 times the number of slides per day with fluorescence technique.(20, 28) However, the use of mercury vapor or halogen lamp as the source of light in the standard fluorescence microscopy technique (FM) is expensive and requires frequent replacement because of their shorter life span making it economically inefficient for use in resource constrained settings. The LED based microscopes (CY and PiLED) on the other hand are structurally built to overcome the hard operational environment of RLS where continuous supply of electricity, sufficient equipment and trained personnel are a frequent challenge. The LED microscopes use lamps that are inexpensive with much longer life span. They are also simple to operate; and no dark room required.

The scanty-positive findings are more commonly seen with ZN technique and least with the FM. This could be attributed to the low-power objective of the fluorescence techniques that allow the field to be seen larger than in the conventional ZN light microscopy. Some of the samples studied may have come from HIV co-infected patients with low-density bacilli yielding scanty AFBs on the conventional ZN microscopy earning a grade of 1+ or more when viewed under the fluorescence techniques. However, the fluorescence techniques recorded the highest numbers of false positives compared to the ZN judging from the reported frequencies in the decision matrix table. This is expected because the low-threshold of the fluorescence techniques derived from their low-power objective could allow naturally fluorescence particles present in the sputum, certain spores, fungi to appear AFB positive which may otherwise be negative on ZN microscopy. These and the fact that significant proportion of the samples studied may have come from HIV co-infected patients could also explain the particularly low specificity of the PiLED technique. Associated HIV infection was believed to account for low specificity of LED-FM microscopy compared to the conventional ZN microscopy in two previous studies in settings with high burden of the disease (29, 30)

As expected, the digestion and decontamination with n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) produces the highest yield of AFB in the sputum exposed to the reagent for 15 minutes. This shows that the manufacturer's recommended exposure time of 15 minutes works well in the hard operational environment of a resource limited setting. The 10 minutes alternative yielded a little less AFB than the 15 minutes duration probably because the time is not enough to allow complete digestion of the thick sputum which allows the release of mycobacteria and their subsequent concentration by centrifugation. The 20 minutes exposure on the other hand might have been prolonged beyond the optimal duration necessary and the toxic effect of the reagent kills not only the contaminating normal flora that may overgrow in cultures making it hard to detect the presence of the mycobacteria but also the mycobacteria itself. The success of this process is dependent on the concentration of the reagent, the exposure time, centrifugation and the temperature selected. In this case all other factors were kept constant and only the exposure time was altered.

The design of this study allows test performance assessment under routine (real) resource constrain settings. The point estimates obtained are more likely to represent the expected outcomes in similar settings with high prevalence of TB. Blinding the readers on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of the direct smear results. However, our study had a few limitations: We did not obtain participants' HIV status and therefore unable to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures. The time taken to read slides was also not reflected in the analysis because only estimates of the time taken to read the slides were recorded since the different technicians that read the slides had different proficiency levels and some accurately read the slides much faster than others.

In summary, the high sensitivity for AFB detection shown by the inexpensive light-emitting diode based Primo Star iLED fluorescence microscope in the hard operational conditions of a resource limited setting underscores the potentials for its scalability to remote clinics in similar settings.. This will increase TB detection and improve TB treatment uptakes in high burden settings with scarce resources. The optimal exposure duration for decontamination of sputum samples prior to examination and culture using the n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) reagent in this setting is 15 minutes.

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**Competing interest**: All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

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## References


1. De Cock KM, Simone PM, Davison V, Slutsker L. The new global health. Emerg Infect Dis. 2013 Aug;19(8):1192-7.
2. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. [Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.
Review]. 2003 May 12;163(9):1009-21.
3. Young DB, Perkins MD, Duncan K, Barry CE, 3rd. Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest. [Research Support, Non-U.S. Gov't
Review]. 2008 Apr;118(4):1255-65.
4. Getahun H, Harrington M, O'Brien R, Nunn P. Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. Lancet. [Review]. 2007 Jun 16;369(9578):2042-9.
5. Somoskovi A, Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M. Lessons from a proficiency testing event for acid-fast microscopy. Chest. [Research Support, U.S. Gov't, P.H.S.]. 2001 Jul;120(1):250-7.
6. Van Deun A, Salim AH, Cooreman E, Hossain MA, Rema A, Chambugonj N, et al. Optimal tuberculosis case detection by direct sputum smear microscopy: how much better is more? Int J Tuberc Lung Dis. [Evaluation Studies]. 2002 Mar;6(3):222-30.
7. Cole EC, Cook CE. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. Am J Infect Control. [Review]. 1998 Aug;26(4):453-64.
8. Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin Microbiol Rev. [Review]. 2011 Apr;24(2):314-50.
9. Monkongdee P, McCarthy KD, Cain KP, Tasaneeyapan T, Nguyen HD, Nguyen TN, et al. Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. Am J Respir Crit Care Med. [Evaluation Studies
Research Support, U.S. Gov't, Non-P.H.S.]. 2009 Nov 1;180(9):903-8.
10. Cheng S, Chen W, Yang Y, Chu P, Liu X, Zhao M, et al. Effect of Diagnostic and Treatment Delay on the Risk of Tuberculosis Transmission in Shenzhen, China: An Observational Cohort Study, 1993-2010. PLoS One. 2013;8(6):e67516.
11. Tadesse T, Demissie M, Berhane Y, Kebede Y, Abebe M. Incidence of smear-positive tuberculosis in Dabat, northern Ethiopia. Int J Tuberc Lung Dis. [Research Support, U.S. Gov't, Non-P.H.S.]. 2013 May;17(5):630-5.
12. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. [Comparative Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.
Review]. 2006 Sep;6(9):570-81.

13. Hooja S, Pal N, Malhotra B, Goyal S, Kumar V, Vyas L. Comparison of Ziehl Neelsen & Auramine O staining methods on direct and concentrated smears in clinical specimens. Indian J Tuberc. [Comparative Study]. 2011 Apr;58(2):72-6.
14. Prasanthi K, Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. Indian J Med Microbiol. [Comparative Study]. 2005 Jul;23(3):179-81.
15. Abe C. [Standardization of laboratory tests for tuberculosis and their proficiency testing]. Kekkaku. [Review]. 2003 Aug;78(8):541-51.
16. Allen BW. Comparison of three methods for decontamination of faeces for isolation of Mycobacterium tuberculosis. Tubercle. [Comparative Study]. 1991 Sep;72(3):214-7.
17. Krasnow I, Wayne LG. Comparison of methods for tuberculosis bacteriology. Appl Microbiol. [Comparative Study]. 1969 Nov;18(5):915-7.
18. Selvakumar N, Rahman F, Rajasekaran S, Narayanan PR, Frieden TR. Inefficiency of 0.3% carbol fuchsin in ziehl-neelsen staining for detecting acid-fast bacilli. J Clin Microbiol. [Comparative Study
Evaluation Studies
Research Support, Non-U.S. Gov't]. 2002 Aug;40(8):3041-3.
19. http://www.uphs.upenn.edu/bugdrug/antibiotic_manual/IUATLD_afb%20microscopy_guide.pdf. Accessed last on December 25.
20. Lehman LG, Ngapmen Yamadji AL, Ngo Sack F, Bilong Bilong CF. The CyScope(R) fluorescence microscope, a reliable tool for tuberculosis diagnosis in resource-limited settings. Am J Trop Med Hyg. [Research Support, Non-U.S. Gov't]. 2010 Oct;83(4):906-8.
21. Hawass NE. Comparing the sensitivities and specificities of two diagnostic procedures performed on the same group of patients. Br J Radiol. [Comparative Study]. 1997 Apr;70(832):360-6.
22. Hung NV, Sy DN, Anthony RM, Cobelens FG, van Soolingen D. Fluorescence microscopy for tuberculosis diagnosis. Lancet Infect Dis. [Comment
Letter]. 2007 Apr;7(4):238-9; author reply 9-40.
23. Van Deun A, Chonde TM, Gumusboga M, Rienthong S. Performance and acceptability of the FluoLED Easy module for tuberculosis fluorescence microscopy. Int J Tuberc Lung Dis. [Comparative Study
Evaluation Studies
Research Support, U.S. Gov't, Non-P.H.S.]. 2008 Sep;12(9):1009-14.
24. Ulukanligil M, Aslan G, Tasci S. A comparative study on the different staining methods and number of specimens for the detection of acid fast bacilli. Mem Inst Oswaldo Cruz. [Comparative Study]. 2000 Nov-Dec;95(6):855-8.
25. Cuevas LE, Al-Sonboli N, Lawson L, Yassin MA, Arbide I, Al-Aghbari N, et al. LED fluorescence microscopy for the diagnosis of pulmonary tuberculosis: a multi-country cross-sectional evaluation. PLoS Med. [Controlled Clinical Trial
Multicenter Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.]. 2011 Jul;8(7):e1001057.
26. Habtamu M, van den Boogaard J, Ndaro A, Buretta R, Irongo CF, Lega DA, et al. Light-emitting diode with various sputum smear preparation techniques to diagnose tuberculosis. Int J Tuberc Lung Dis. [Comparative Study
Research Support, Non-U.S. Gov't]. 2012;16(3):402-7.
27. Marais BJ, Brittle W, Painczyk K, Hesseling AC, Beyers N, Wasserman E, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clin Infect Dis. [Comparative Study

Evaluation Studies]. 2008 Jul 15;47(2):203-7.
28. Ba F, Rieder HL. A comparison of fluorescence microscopy with the Ziehl-Neelsen technique in the examination of sputum for acid-fast bacilli. Int J Tuberc Lung Dis. [Comparative Study]. 1999 Dec;3(12):1101-5.
29. Cattamanchi A, Davis JL, Worodria W, den Boon S, Yoo S, Matovu J, et al. Sensitivity and specificity of fluorescence microscopy for diagnosing pulmonary tuberculosis in a high HIV prevalence setting. Int J Tuberc Lung Dis. [Comparative Study
Evaluation Studies
Research Support, N.I.H., Extramural]. 2009 Sep;13(9):1130-6.
30. Chaidir L, Parwati I, Annisa J, Muhsinin S, Meilana I, Alisjahbana B, et al. Implementation of LED fluorescence microscopy for diagnosis of pulmonary and HIV-associated tuberculosis in a hospital setting in Indonesia. PLoS One. [Research Support, Non-U.S. Gov't]. 2013;8(4):e61727.

**Abstract**

**Objectives:** The light-emitting diode (LED) ~~florescence~~fluorescence microscopy has made acid-fast bacilli (AFB) detection faster and efficient although its optimal performance in resource limited settings is still being studied. We assessed the optimal performances of light and ~~florescence~~fluorescence microscopy in routine conditions of a resource limited setting and evaluated the digestion time for sputum samples for maximum yield of positive cultures.

**Design:** Cross-sectional study

**Setting:** Facility-based involving samples of routine patients receiving tuberculosis treatment and care from the main tuberculosis case referral center in northern Nigeria.

**Participants:** The study included 450 sputum samples from 150 new patients with clinical diagnosis of pulmonary tuberculosis.

**Methods:** The 450 samples were pooled into 150 specimens, examined independently with mercury vapor lamp (FM), LED ~~CyScope~~CysCope® (CY) and Primo Star iLED (PiLED) ~~florescence~~fluorescence microscopies, and with the Ziehl Neelsen (ZN) microscopy to assess the performance of each technique compared to liquid culture. The cultured specimens were decontaminated with Mycoprep (4%NaOH-1%N~~aC~~LAC and 2.9% sodium citrate) for 10, 15 and 20 minutes before incubation in Mycobacterium Growth Incubator Tube (MGIT) system and growth examined for acid-fast bacilli (AFB)

**Results:** Of the 150 specimens examined by direct microscopy: 44 (29%), 60 (40%), 49 (33%) and 64 (43%) were AFB positive by ZN, FM, CY, and iLED microscopies. Digestion of sputum samples for 15min yielded the highest ~~number of AFBs~~mycobacterial growth as illustrated by AFB growth in 72 (48%), 81 (54%) and 68 (45%) cultures with sputum samples digested~~econtaminated~~ for~~at~~ 10, 15 and 20 minutes respectively.

**Conclusions:** In routine laboratory ~~conditions~~ conditions of a~~in~~ resource-limited setting, our study has demonstrated the superiority of ~~the florescence~~ fluorescence microscopy over the conventional ZN technique.~~and the Primo Star iLED microscopy~~. Digestion of sputum samples for 15minutes yielded more positive cultures

**Keywords:** Tuberculosis, M~~m~~icrobiology, Respiratory infections

**Optimizing Mycobacterium tuberculosis detection in resource limited settings**

## Introduction

Globally, an estimated 1.4 million death occurred in 2011 as a result of infection with tuberculosis, one-fourth of the death was associated with HIV infection and most of it in resource limited settings (1) where the burden of HIV infection is high. In Africa, TB is the leading cause of death among HIV infected persons as the continent harbors eighty percent of the world HIV-TB cases (2). Nigeria and the Republic of South Africa are among the top five TB burden nations of the world. Sensitive, specific, and inexpensive point-of-care diagnostic tools for rapid TB detection are lacking posing challenges to the optimal diagnosis of TB in resource limited settings (RLS), particularly among persons with HIV infections whose sputum specimen often lack sufficient quantity of acid-fast bacilli (AFB) detectable on routine smear examination. (3, 4)

The simplicity, inexpensiveness and predictive power of the Ziehl Neelsen sputum smear microscopy make it the applicable laboratory diagnostic tool of choice for tuberculosis in resource limited settings. (5) A~~When~~ properly done~~, a~~ positive sputum-smear is highly predictive of active TB (6, 7). Unfortunately, the~~its~~ sensitivity of sputum-smear microscopy is low and its performance is often affected by lack of proper maintenance of equipment; trained manpower; and quality assurance system in settings where resources are scarce. (8) Even in centers with good quality assurance measures, smear microscopy~~AFB~~ detects only 40-50% of TB cases compared to about 80% yield by culture (9). However, it remains the only available tool for bacteriologic diagnosis of TB in most RLS. Culture techniques are highly sensitive and specific, but the cost, technical complexity and time delay before results are produced make culture not ~~un~~scalable for rapid detection and treatment of tuberculosis. Recent advances have made it possible to use molecular technology to detect ~~AFB~~Mycobacterial d~~D~~eoxyribonucleic acid (DNA)~~s~~ with great accuracy. However these technologies still require a fairly developed laboratory with a reliable source of electricity.

Detection of smear positive cases is the priority in TB control programs. These cases are infectious and contribute substantially to the transmission of the disease. (10, 11) In recent years, several new laboratory techniques have been developed to significantly improve the ability to diagnose active TB in populations. Fluorescence microscopy improves the sensitivity of sputum-smear detection and has good specificity relative to the conventional ZN method. Inexpensive light-emitting diode-based (LED) fluorescence microscopes have been developed that make microscopy of smears stained by Auramine ~~florescence~~fluorescence dye easier, cheaper and faster with potentials for scalability to remote clinics in RLS. (5, 12-14) And, they can be battery operated as well.

This study compared the performance of the standard but more expensive mercury vapor lamp (FM) ~~flo-rescence~~fluorescence microscope; the newer, less expensive LED based ~~CyScope~~CysCope® (CY) (Partec, Görlitz, Germany); and the Primo Star iLED (PiLED) (Carl Zeiss Inc. Germany) ~~florescence~~fluorescence microscopes in comparison to the conventional light ( ZN) microscope against the gold (reference) standard of liquid culture in automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems) for the detection of AFB from sputum samples of patients with clinical pulmonary tuberculosis (TB). In addition, we evaluated the optimal digestion time with *Mycoprep (4%NaOH-1%NLAC and 2.9% sodium citrate)* for liquid culture in this population. Prolonged decontamination time is reported to reduce the number of viable bacilli and different digestion methods yield variable number of positive cultures (15-17). ~~processing conditions for liquid culture in the study population.~~

**Methods**

*Study population*

Three sputum samples (spot-morning-spot) were collected from 150 TB treatment naïve, consecutive patients with clinical diagnosis of pulmonary tuberculosis at the National TB and Leprosy Training Center (NTBLTC), Zaria, Nigeria. The spot-morning-spot sample collection was the standard routine for the diagnosis of TB in Nigeria at the time of the study. An overview of the study design flow chart is provided in figure 1. Ethical review was waived because in the opinion of the study center review committee there was no potential risk to participants' safety, privacy or confidentiality since there was no formal contact between investigators and participants either directly (interview, questionnaires, etc.) or indirectly (medical records, personal identifiers etc.). The sputum specimens provided for routine clinical care services were completely anonymized before they were analyzed for the study and there was no risk that the pooled samples can be de-anonymized through data linkages. ~~This study was conducted within the routine laboratory protocols for TB control and the investigators had no direct contact with the patients. Samples were completely de-identified after the initial results were obtained for clinical care and no additional clinical data were collected.~~ This study was conducted from July to September of 2009.

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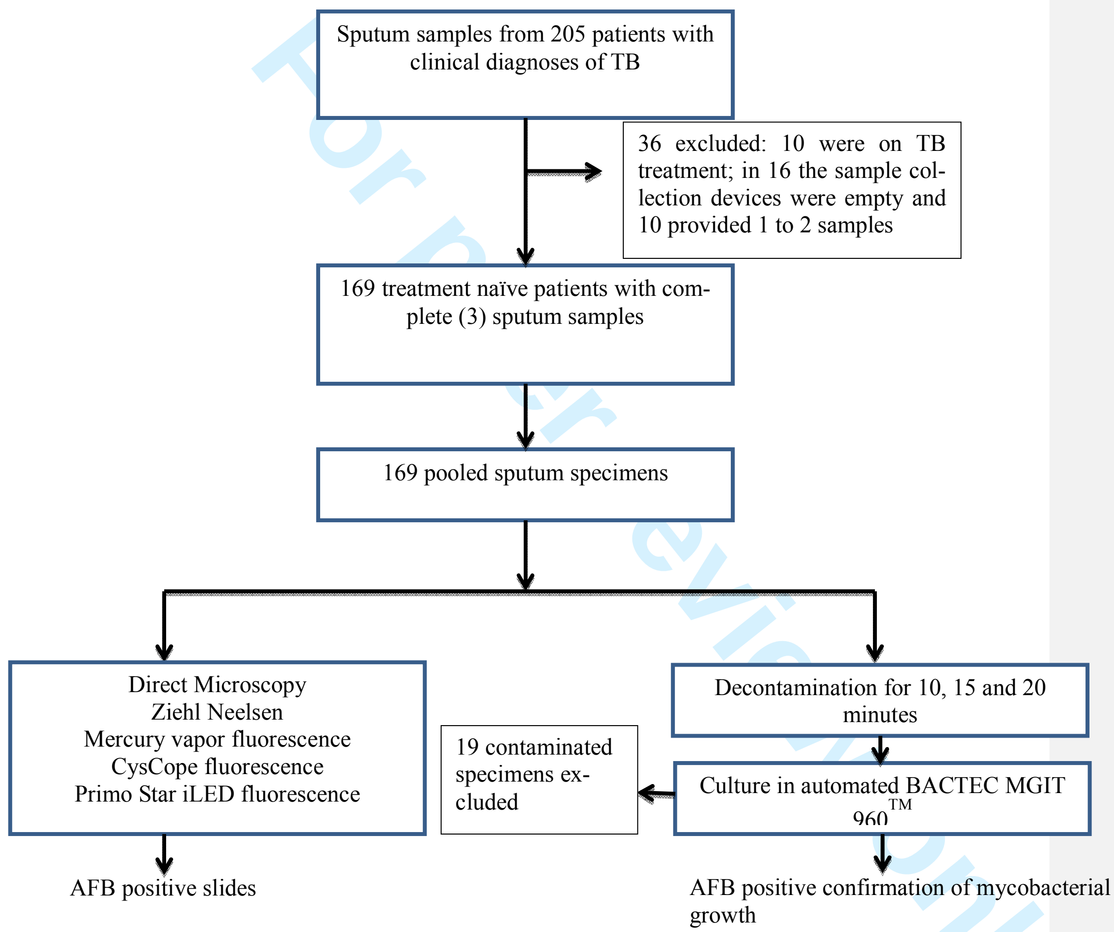


Figure 1. Flow chart for the study design involving 450 sputum samples from 150 patients with clinical TB

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*Smear Microscopy*

The three sputum samples from each patient were pooled. Four direct smears were made from each of the pooled sputum samples with applicator sticks on clean grease-free slides; measuring 2 cm x 3 cm, and not too thick. The first slide was stained with Ziehl Neelsen (ZN) stain according to the WHO recommended protocol described elsewhere (18). Briefly, the smear was air dried and fixed by gently passing it over a flame 2-3 times. One percent strong carbol-fuchsin was applied to the slide and heated with a Bunsen flame intermittently 3 times and allowed to stain for 15 minutes. The stain was rinsed off with gentle tap water and 3% acid alcohol was applied on the smear for 3 minutes. Methylene blue (0.3%) was then added for up to 1 minute and rinsed off with tap water. Positive and negative controls were included in the process. Slide e~~E~~xamination~~ of the slide~~ was done with light microscope at 1,000x magnification and the AFBs identified were graded according to the International Union Against Tuberculosis and Lung Disease (IUATLD) and the World Health Organization ~~/~~(WHO) smear grading scale(19)~~ (guideline)~~.

The second, third and fourth slides were air-dried, heat-fixed and stained with Auramine O-phenol for 15 minutes rinsed with tap water, decolorized with 1 percent acid alcohol for 2 minutes then rinsed with water and counterstained with 0.1 percent potassium permanganate for 2 minutes. The slides were rinsed with water then allowed to~~ drain~~ dry in air before examination under FM, CY and PiLED microscopes respectively. The AFBs were visualized and graded at magnifications of 200× and 400× according to a protocol describe by Lehman (20). A total of 100 microscopic fields were examined before a slide is recorded as positive (if at least an AFB was seen) or negative (if no AFB was seen). ~~Using the **IUATLD/WHO smear grading scale (guideline),**~~ ~~P~~P~~p~~ositive smears were graded as scanty (actual) if there were: 1-9 AFBs in at least 100 fields; +1: 10-99 in 100 fields; +2: 1-10 per field in at least 50 fields; and +3: if there were more than 10 per field in at least 20 fields. Absence of any AFB was graded as negative smear. Each slide was examined by three independent readers to ascertain the presence of AFB and grade positive smears. The slide readers were blinded on the clinical and~~or~~ laboratory diagnose~~i~~s of the subjects whose samples were studied~~examined~~.

*Specimen culture*

Each of the 150 pooled sputum specimens were decontaminated with BD Mycoprep™ (Beckton Dickinson Diagnostic Systems, Sparks, Maryland, USA) which consists of 1% N-acetyl-L-cysteine (NALC), 4% sodium hydroxide and 2.9% sodium citrate. An equal amount of Mycoprep was added to the sputum, homogenized and allowed to act for 10, 15 and 20 minutes respectively. After the respective digestion period, phosphate buffered saline was added to stop the digestion and decontamination reaction. This was then centrifuged under refrigerated conditions with uniform centrifugation time of 15 minutes and a Relative Centrifugal force (RCF) of 3000 x g. The supernatants were discarded and the sediments re-suspended with 1 ml of phosphate buffered saline to make a final volume of 2 ml. Then 0.5 ml of each was inoculated into Mycobacterium Growth Indicator Tubes (MGIT) and incubated in the automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems), which monitors growth. Positive and negative control culture tubes were also set up along each test using *Mycobactreium tuberculosis* H37Ra (ATCC 25177 – attenuated strain) and sterial distilled water respectively.

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The cultures that indicated positive growth were removed from the machine and the presence of AFB confirmed by ZN smear microscopy. Those that do not indicate growth after 42 days were classified as negatives as recommended by the manufacturer and as was the standard practice. All cultures were done at Zankli Research laboratory while all microscopy were performed at the NTBLTC, Zaria to minimize interference within ~~in order not to influence~~ the outcome of culture or direct microscopy results.

*Statistical analysis*

The raw data generated from the smear microscopic examinations of the 150 matched samples by the four different microscopes as well as the data generated after same samples were cultured following decontaminations (digestions) at 10, 15 and 20 minutes were organized into cell counts in which descriptive values were provided along with frequencies and proportions. Measures of accuracy for the different smear examinations: sensitivity, specificity, positive and negative predictive values were evaluated through decision matrix tables while concordance and disagreements between the microscopic examination by each of the three ~~florescence~~fluorescence microscopes and the conventional light (ZN) microscope based on the final classification of samples by the gold standards (outcomes MGIT cultures) were examined in matched sample tables for positive and negative final outcomes as previously recommended for comparative analysis of diagnostic procedures performed on same samples (patients) (21). Comparison between the ~~florescence~~fluorescence microscopies to the conventional ZN microscopy for differences in performance together with their 95% confidence intervals was done using the McNemar's test. Significance of association was set at 0.05 alpha and all probabilities were two tailed.

**Results**

Of the 150 matched (paired) samples, AFBs were detected in: 44 (29%) of the ZN smears examined under light microscope; 60 (40%) of the AR smears examined with FM; 49 (33%) of the AR smears examined with CY;

and 64 (44%) AR smears examined with PiLED ~~floresecence~~fluorescence microscopes. MGIT cultures of the 150 specimen yielded 72 (48%), 81 (54%) and 68 (45%) AFB confirmed growths after decontamination for 10 (A), 15 (B) and 20 (C) minutes respectively prior to incubation.~~positives in the group decontaminated for 10 minutes (A) prior to incubation; 81 (54%) in those decontaminated for 15 minutes (B) and 68 (45%) in those with 20 minutes (C) decontamination time.~~ ,).

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~~The contamination rate of the culture method decontamination times were 10 min (6%), 15 min (4%) and 20 min (7%). Of the direct microscopy techniques, AR smears examined by the PilED gave the highest yield and was surprising comparable to cultures from 10 and 20 minutes digestion. As expected the ZN detected the list number of positive slide so did the CY~~.

Among the positive AFB tests graded, ZN light microscopy produced the highest proportion of samples with scanty AFBs on examination 21/44 (48%), followed by PiLED ~~floresecence~~fluorescence microscopy 12/64 (19%), then CY 8/49 (16%) and FM had the least scanty AFB positive counts~~results~~ 3/60 (5%). However, after~~when~~ the samples with scanty positive AFBs where incubated in A, B and C MGIT culture groups, the proportions of confirmed mycobacterial growths ~~by the cultures~~ in the sequence above were as follows: (i) ZN: 14/21 (67%), 9/21 (42%) and 11/21 (52%); (ii) PiLED: 5/12 (42%), 7/12 (58%) and 5/12 (42%); (iii) CY: 6/8 (75%), 4/8 (50%) and 3/8 (38%); lastly (iv) FM: 0/3 (0%), 1/3 (33%) and 1/3 (33%). A summary of the positive AFB outcomes from the direct smear examinations by microcopy types, grades and the proportions of graded smears confirmed by the MGIT cultures were provided in Table 1.

Table 1. Proportions of positive graded direct smears confirmed by cultured specimen decontaminated at 10 (A), 15 (B) and 20(C) minutes obtained from 150 matched samples

| Direct | Grade | Frequency | MGIT cultured specimen (Gold standards) |
|---|---|---|---|

| smear (DS) | | | A | | B | | C | |
|---|---|---|---|---|---|---|---|---|
| | | | n | % | n | % | n | % |
| ZN | scanty | 21 | 14 | 66.7 | 9 | 42.1 | 11 | 52.4 |
| | +1 | 13 | 12 | 92.3 | 11 | 84.6 | 10 | 76.9 |
| | +2 | 5 | 5 | 100.0 | 4 | 80.0 | 5 | 100.0 |
| | +3 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| FM | Scanty | 3 | 0 | 0.0 | 1 | 33.3 | 1 | 33.3 |
| | +1 | 34 | 24 | 70.6 | 19 | 55.9 | 17 | 50.0 |
| | +2 | 18 | 16 | 88.9 | 12 | 66.7 | 13 | 72.2 |
| | +3 | 5 | 4 | 80.0 | 4 | 80.0 | 4 | 80.0 |
| CY | Scanty | 8 | 6 | 75.0 | 4 | 50.0 | 3 | 37.5 |
| | +1 | 30 | 22 | 73.3 | 18 | 60.0 | 19 | 63.3 |
| | +2 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| | +3 | 6 | 6 | 100.0 | 6 | 100.0 | 6 | 100.0 |
| PiLED | Scanty | 12 | 5 | 41.7 | 7 | 58.3 | 5 | 41.7 |
| | +1 | 36 | 22 | 61.1 | 20 | 55.6 | 18 | 50.0 |
| | +2 | 13 | 13 | 100.0 | 10 | 76.9 | 13 | 100.0 |
| | +3 | 3 | 3 | 100.0 | 2 | 66.7 | 3 | 100.0 |

ZN: Ziehl Neelsen microscopy using the conventional light microscope
FM: ~~Floresecence~~Fluorescence microscopy with mercury vapor lamp microscope
CY: ~~Floresecence~~Fluorescence microscopy with ~~Cyscope~~CysCope light emitting diode microscope
PiLED: ~~Floresecence~~Fluorescence microscopy with Primo Star iLED light emitting diode microscope

A comparison of ~~the outcomes~~ ~~of~~ the direct smear tests results with the final culture outcomes~~results~~ to measure the performances of the direct smear techniques against the gold ~~standards showed~~standards showed that of the 44 AFB positive samples identified by the ZN microscopy; 36 (81.8%), 29 (65.9%) and 31 (70.5%) were culture confirmed at 10, 15 and 20 minutes decontamination times respectively. Similarly, of the 60, 49 and 64 AFB positive samples identified by the FM, CY and PiLED microscopy techniques the frequencies and proportions of the culture confirmed were: 44 (73.3%) 36 (60.0%) and 35 (58.3%) for FM; 39 (79.6%), 33 (67.3%) and 33 (67.3%) for CY; and 43 (67.2%) 39 (56.3%) and 39 (56.3%) for PiLED at decontamination times of 10, 15 and 20 minutes respectively. There were ~~near~~ near identical frequency distributions for the true positive (TP), false positive (FP), false negative (FN) and true negative (TN) for the ZN and CY microscopy techniques while the~~. The~~ FM microscopy ~~however~~ yielded a pattern of distribution similar to that of~~e~~ PiLED techniques as shown in the decision matrix of Table 2.

Table 2. Decision matrix for the evaluation of performance of the direct smear methods against the different gold standards for the 150 matched samples

| Direct Smear (DS) | DS Outcome | Gold standards: MGIT cultures | | | | | |
|---|---|---|---|---|---|---|---|
| | | A : culture positive =72 | | B: culture positive =81 | | C: culture positive = 68 | |
| | | P | N | P | N | P | N |
| ZN | P | 36 | 08 | 29 | 15 | 31 | 13 |
| DS+ = 44 | N | 36 | 70 | 52 | 54 | 37 | 69 |
| FM | P | 44 | 16 | 36 | 24 | 35 | 25 |
| DS+ =60 | N | 28 | 62 | 45 | 45 | 33 | 57 |
| CY | P | 39 | 10 | 33 | 16 | 33 | 16 |
| DS+ =49 | N | 33 | 68 | 48 | 53 | 35 | 66 |
| PiLED | P | 43 | 21 | 39 | 25 | 39 | 25 |
| DS+ =64 | N | 29 | 57 | 42 | 44 | 29 | 57 |

Culture specimen decontaminated by NaOH-NA~~aC~~LC : A for 10 minutes; B for 15minutes; C for 20 minutes
DS+ = number of samples (out of 150 samples) that were AFB positive by direct smear
P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor ~~florescence~~fluorescence microscopy; CY=~~CyScope~~CysCope ~~florescence~~fluorescence microscopy and PiLED= Primo Star iLED ~~florescence~~fluorescence microscopy

The levels of agreement and differences between the convention ZN microscopy and each of the ~~florescence~~fluorescence microscopy techniques with respect to the final culture results were demonstrated~~scribed~~ by the concordance and discordance cells displayed in Table 3. Cells were discordant if the outcomes of the two techniques disagreed otherwise they were concordant. The statistical significance of the difference in the performance of the techniques compared was derived from the discordant cell. The sum of the frequencies of the discordant cells for example that explained the differences between ZN and CY were 7, 4 and 2 for the positive culture results which showed less consistent divergence compared to 6, 6, and 7 for the negative culture results of the same techniques. The sum of the discordant frequencies between ZN and FM, and between ZN and PiLED were higher relative to those between ZN and CY.

Table 3: Matched sample frequency cells for the agreement and discordance between the standard of care (ZN smear) and FM, CY and PiLED smears on the culture positive and negative specimens

| | | | FM | | CY | | PiLED | |
|---|---|---|---|---|---|---|---|---|
| Culture Positive | | | P | N | P | N | P | N |
| A: n= 72 | ZN | P | 35 | 1 | 34 | 2 | 35 | 1 |
| | | N | 9 | 27 | 05 | 31 | 08 | 28 |
| B: n= 81 | ZN | P | 29 | 0 | 29 | 0 | 29 | 0 |
| | | N | 7 | 45 | 4 | 48 | 10 | 42 |
| C: n= 68 | ZN | P | 29 | 2 | 31 | 0 | 29 | 2 |
| | | N | 6 | 31 | 2 | 35 | 10 | 27 |
| Culture Negative | | | | | | | | |
| A: n= 78 | ZN | P | 6 | 2 | 7 | 2 | 9 | 0 |
| | | N | 7 | 63 | 4 | 65 | 12 | 57 |
| B: n= 69 | ZN | P | 11 | 1 | 6 | 2 | 14 | 0 |
| | | N | 10 | 47 | 4 | 57 | 10 | 45 |
| C: n= 82 | ZN | P | 11 | 1 | 10 | 1 | 13 | 0 |
| | | N | 12 | 58 | 6 | 65 | 10 | 59 |

P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor ~~florescence~~fluorescence microscopy; CY=~~CyScope~~CysCope ~~florescence~~fluorescence microscopy and PiLED= Primo Star iLED ~~florescence~~fluorescence microscopy

A: cultured samples decontaminated for 10 minutes; B: cultured samples decontaminated for 15 minutes; C: cultured samples decontaminated for 20 minutes

The~~ sensitivity of the~~ ZN technique was consistently less sensitive~~ess~~ compared to any of the ~~florescence~~fluorescence techniques~~ measured~~ against~~ all the three~~ any of the gold standards. The ~~florescence~~fluorescence method FM was: 11.1%, 8.6% and 5.9; CY was: 4.2%, 4.9% and 3.1% while PiLED was 9.7%, 12.3%, and 11.8% more sensitive than ZN microscopy ( $P_{fm}$= 0.027, 0.023, 0.289; $P_{cy}$ = 0.450, 0.134, 0.479 and $P_{iled}$ =0.046, 0.004, 0.043). Conversely, the FM microscopy was: 10.2%, 13.1% and 14.6%; CY was: 2.5% 2.5% and

3.6%; PiLED was: 16.6%, 1.4% and 14.6% less specific compared to the ZN microscopy ( $P_{fm}$= 0.182, 0.016, 0.006; $P_{cy}$ = 0.684, 0.684, 0.131 and $P_{iled}$ =0.006, 0.131, 0.004) and also less predictive compared to the ZN (Table 4)

Table 4. Summary measures of performance of the direct smear diagnostic tests for 150 matched samples and the statistical difference in sensitivity and specificity of FM, CY and PiLED compared to ZN

| Gold Stand | Direct Smear | Sn | Sp | PPV | NPV | SnD [95% CI] | p-value* | SpD [95% CI] | p-value* |
|---|---|---|---|---|---|---|---|---|---|
| A | ZN | 50.0 | 89.7 | 81.8 | 66.0 | | | | |
| | FM | 61.1 | 79.5 | 73.3 | 68.9 | 11.1 [3.0, 19.0] | 0.027 | 10.2 [2.5, 17.4] | 0.182 |
| | CY | 54.2 | 87.2 | 79.6 | 67.3 | 4.2 [-2.9, 11.3] | 0.450 | 2.5 [-3.6, 8.6] | 0.684 |
| | PiLED | 59.7 | 73.1 | 67.2 | 66.3 | 9.7 [1.9, 17.5] | 0.046 | 16.6 [8.6, 24.6] | 0.001 |
| B | ZN | 35.8 | 78.3 | 65.9 | 50.9 | | | | |
| | FM | 44.4 | 65.2 | 60.0 | 50.0 | 8.6 [2.4, 14.8] | 0.023 | 13.1 [3.9, 22.1] | 0.016 |
| | CY | 40.7 | 76.8 | 67.3 | 52.5 | 4.9 [0.2, 9.6] | 0.134 | 2.5 [-4.4, 9.4] | 0.684 |
| | PiLED | 48.1 | 63.8 | 60.9 | 51.2 | 12.3 [4.9, 19.6] | 0.004 | 1.4 [-5.5, 7.9] | 0.683 |
| C | ZN | 45.6 | 84.1 | 70.5 | 65.1 | | | | |
| | FM | 51.5 | 69.5 | 58.3 | 63.3 | 5.9 [-2.1, 13.9] | 0.289 | 14.6 [6.6, 22.6] | 0.006 |
| | CY | 48.5 | 80.5 | 67.3 | 65.3 | 3.1 [-0.9, 7.1] | 0.479 | 3.6 [-2.6, 9.8] | 0.131 |
| | PiLED | 57.4 | 69.5 | 60.9 | 66.3 | 11.8 [2.2, 21.4] | 0.043 | 14.6 [7.5, 21.7] | 0.004 |

Sn: Sensitivity; Sp: Specificity; PPV: positive predictive value; NPV; Negative predictive value
SnD: Sensitivity difference; percentage points above ZN performance
SpD: Specificity difference; percentage points below ZN performance
CI: Confidence Interval; *: Chi-square p-value generated from McNemar's test

**Discussion**

The findings of this study further confirm the previously reported superior performance of ~~florescence~~fluorescence microscopy over the conventional ZN technique for AFB detection (22-24). The direct smear staining sensitivities of FM and PiLED ~~florescence~~fluorescence techniques are on the average 10% more sensitive than the standard of care (ZN method). The reverse is however true for specificity, the ~~flo-rescence~~fluorescence methods are less specific than ZN by similar percentage points and these differences are statistically significant. Similar trends of high sensitivity and low specificity were observed in the performance of ~~florescence~~fluorescence microscopy in comparison to the conventional Ziehl Neelsen technique (24-26) Within the ~~florescence~~fluorescence techniques FM and PiLED have similar performance measures with little or no differences which is in agreement with the reported findings by Marais et al.(27) The CY technique appears to be less sensitive compared to FM and PiLED but on the average have 4% staining sensitivity advantage over the conventional ZN. Similar findings were reported from a resource constrained setting with comparable optimal technical conditions in which such differences were found to be marginally significant statistically. (20). However, both CY and ZN staining techniques have higher specificities compared to FM and PiLED across all the three reference (gold) standards used.

Although less sensitive, the ZN staining seems to be the most predictive: a positive test has the highest chance of being a true case of TB while a negative test is less likely to harbor the disease. The CY has similar predictive probabilities; however, these measures are affected by disease burden. In high prevalence settings, the positive predictive value of a test increases because it is more likely that those who test positive truly have disease than if the test was performed in settings with low prevalence. Likewise, in settings with low prevalence it is more likely that those who test negative truly do not have the disease. In contrast, sensitivity and specificity measures are less susceptible to~~not~~ influence~~d~~ ~~by disease~~by disease prevalence. The search for an optimal tuberculosis diagnostic test is motivated by the unacceptably low sensitivity of the conventional ZN smear microscopy over which ~~florescence~~fluorescence microscopy has shown consistent superiority. In this setting, the TB prevalence is high and may be driven by HIV. Priority should therefore be given to sensitivity over predictive value since failure to detect TB in patients co-infected with HIV is more likely to increase morbidity and mortality among the HIV infected persons than false detection of TB.

In addition to improved TB case detection in samples with low-density bacilli, which is aided by its low-power objective, previous studies reported that ~~florescence~~fluorescence microscopy requires less than 25% of the time taken to read slides using the conventional ZN technique-meaning a microscopist can examine 4 times the number of slides per day with ~~florescence~~fluorescence technique.(20, 28) However, the use of mercury vapor or halogen lamp as the source of light in the standard ~~florescence~~fluorescence microscopy technique (FM) is expensive and requires frequent replacement because of their shorter life span making it economically inefficient for use in resource constrained settings. The LED based microscopes (CY and PiLED) on the other hand are structurally built to overcome the hard operational environment of RLS where continuous supply of electricity, sufficient equipment and trained personnel are a frequent challenge. The LED microscopes use lamps that are inexpensive with much longer life span. They are also simple to operate; and no dark room required.

The scanty-positive findings are more commonly seen with ZN technique and least with the FM. This could be attributed to the low-power objective of the ~~florescence~~fluorescence techniques that allow the field to be seen larger than in the conventional ZN light microscopy. Some of the samples studied may have come from HIV co-infected patients with low-density bacilli yielding scanty AFBs on the conventional ZN microscopy earning a grade of 1+ or more when viewed under the ~~florescence~~fluorescence techniques. However, the ~~florescence~~fluorescence techniques recorded the highest numbers of false positives compared to the ZN judging from the reported frequencies in the decision matrix table. This is expected because the low-threshold of the ~~florescence~~fluorescence techniques derived from their low-power objective could allow naturally ~~florescence~~fluorescence particles present in the sputum, certain spores, fungi to appear AFB positive which may otherwise be negative on ZN microscopy. These and the fact that significant proportion of the samples studied may have come from HIV co-infected patients could also explain the particularly low specificity of the PiLED technique. Associated HIV infection was believed to account for low specificity of LED-FM microscopy compared to the conventional ZN microscopy in two previous studies in settings with high burden of the disease (29, 30)

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As expected, the digestion and decontamination with n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) produces the highest yield of AFB in the sputum exposed to the reagent for 15 minutes. This shows that the manufacturer's recommended exposure time of 15 minutes works well in the hard operational environment of a resource limited setting. The 10 minutes alternative yielded a little less AFB than the 15 minutes duration probably because the time is not enough to allow complete digestion of the thick sputum which allows the release of mycobacteria and their subsequent concentration by centrifugation. The 20 minutes exposure on the other hand might have been prolonged beyond the optimal duration necessary and the toxic effect of the reagent kills not only the contaminating normal flora that may overgrow in cultures making it hard to detect the presence of the mycobacteria but also the mycobacteria itself. The success of this process is dependent on the concentration of the reagent, the exposure time, centrifugation and the temperature selected. In this case all other factors were kept constant and only the exposure time was altered.

The design of this study allows test performance assessment under routine (real) resource constrain settings. The point estimates obtained are more likely to represent the expected outcomes in similar settings with high prevalence of TB. Blinding the readers on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of the direct smear results. However, our study had a few limitations: We did not obtain participants' HIV status and therefore unable to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures. The time taken to read slides was also not reflected in the analysis because only estimates of the time taken to read the slides were recorded since the different technicians that read the slides had different proficiency levels and some accurately read the slides much faster than others.

In summary, the high sensitivity for AFB detection shown by the inexpensive light-emitting diode based Primo Star iLED ~~florescence~~fluorescence microscope in the hard operational conditions of a resource limited setting underscores the potentials for its scalability to remote clinics in similar settings.. This will increase TB detection and improve TB treatment uptakes in high burden settings with scarce resources. The optimal exposure duration for decontamination of sputum samples prior to examination and culture using the n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) reagent in this setting is 15 minutes.

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## References

1. De Cock KM, Simone PM, Davison V, Slutsker L. The new global health. Emerg Infect Dis. 2013 Aug;19(8):1192-7.
2. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. [Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.
Review]. 2003 May 12;163(9):1009-21.
3. Young DB, Perkins MD, Duncan K, Barry CE, 3rd. Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest. [Research Support, Non-U.S. Gov't
Review]. 2008 Apr;118(4):1255-65.
4. Getahun H, Harrington M, O'Brien R, Nunn P. Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. Lancet. [Review]. 2007 Jun 16;369(9578):2042-9.
5. Somoskovi A, Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M. Lessons from a proficiency testing event for acid-fast microscopy. Chest. [Research Support, U.S. Gov't, P.H.S.]. 2001 Jul;120(1):250-7.
6. Van Deun A, Salim AH, Cooreman E, Hossain MA, Rema A, Chambugonj N, et al. Optimal tuberculosis case detection by direct sputum smear microscopy: how much better is more? Int J Tuberc Lung Dis. [Evaluation Studies]. 2002 Mar;6(3):222-30.
7. Cole EC, Cook CE. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. Am J Infect Control. [Review]. 1998 Aug;26(4):453-64.
8. Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin Microbiol Rev. [Review]. 2011 Apr;24(2):314-50.
9. Monkongdee P, McCarthy KD, Cain KP, Tasaneeyapan T, Nguyen HD, Nguyen TN, et al. Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. Am J Respir Crit Care Med. [Evaluation Studies
Research Support, U.S. Gov't, Non-P.H.S.]. 2009 Nov 1;180(9):903-8.

10. Cheng S, Chen W, Yang Y, Chu P, Liu X, Zhao M, et al. Effect of Diagnostic and Treatment Delay on the Risk of Tuberculosis Transmission in Shenzhen, China: An Observational Cohort Study, 1993-2010. PLoS One. 2013;8(6):e67516.
11. Tadesse T, Demissie M, Berhane Y, Kebede Y, Abebe M. Incidence of smear-positive tuberculosis in Dabat, northern Ethiopia. Int J Tuberc Lung Dis. [Research Support, U.S. Gov't, Non-P.H.S.]. 2013 May;17(5):630-5.
12. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. [Comparative Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.
Review]. 2006 Sep;6(9):570-81.
13. Hooja S, Pal N, Malhotra B, Goyal S, Kumar V, Vyas L. Comparison of Ziehl Neelsen & Auramine O staining methods on direct and concentrated smears in clinical specimens. Indian J Tuberc. [Comparative Study]. 2011 Apr;58(2):72-6.
14. Prasanthi K, Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. Indian J Med Microbiol. [Comparative Study]. 2005 Jul;23(3):179-81.
15. Abe C. [Standardization of laboratory tests for tuberculosis and their proficiency testing]. Kekkaku. [Review]. 2003 Aug;78(8):541-51.
16. Allen BW. Comparison of three methods for decontamination of faeces for isolation of Mycobacterium tuberculosis. Tubercle. [Comparative Study]. 1991 Sep;72(3):214-7.
17. Krasnow I, Wayne LG. Comparison of methods for tuberculosis bacteriology. Appl Microbiol. [Comparative Study]. 1969 Nov;18(5):915-7.
18. Selvakumar N, Rahman F, Rajasekaran S, Narayanan PR, Frieden TR. Inefficiency of 0.3% carbol fuchsin in ziehl-neelsen staining for detecting acid-fast bacilli. J Clin Microbiol. [Comparative Study
Evaluation Studies
Research Support, Non-U.S. Gov't]. 2002 Aug;40(8):3041-3.
19. http://www.uphs.upenn.edu/bugdrug/antibiotic_manual/IUATLD_afb%20microscopy_guide.pdf. Accessed last on December 25.
20. Lehman LG, Ngapmen Yamadji AL, Ngo Sack F, Bilong Bilong CF. The CyScope(R) fluorescence microscope, a reliable tool for tuberculosis diagnosis in resource-limited settings. Am J Trop Med Hyg. [Research Support, Non-U.S. Gov't]. 2010 Oct;83(4):906-8.
21. Hawass NE. Comparing the sensitivities and specificities of two diagnostic procedures performed on the same group of patients. Br J Radiol. [Comparative Study]. 1997 Apr;70(832):360-6.
22. Hung NV, Sy DN, Anthony RM, Cobelens FG, van Soolingen D. Fluorescence microscopy for tuberculosis diagnosis. Lancet Infect Dis. [Comment
Letter]. 2007 Apr;7(4):238-9; author reply 9-40.
23. Van Deun A, Chonde TM, Gumusboga M, Rienthong S. Performance and acceptability of the FluoLED Easy module for tuberculosis fluorescence microscopy. Int J Tuberc Lung Dis. [Comparative Study
Evaluation Studies
Research Support, U.S. Gov't, Non-P.H.S.]. 2008 Sep;12(9):1009-14.
24. Ulukanligil M, Aslan G, Tasci S. A comparative study on the different staining methods and number of specimens for the detection of acid fast bacilli. Mem Inst Oswaldo Cruz. [Comparative Study]. 2000 Nov-Dec;95(6):855-8.

25. Cuevas LE, Al-Sonboli N, Lawson L, Yassin MA, Arbide I, Al-Aghbari N, et al. LED fluorescence microscopy for the diagnosis of pulmonary tuberculosis: a multi-country cross-sectional evaluation. PLoS Med. [Controlled Clinical Trial
Multicenter Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.]. 2011 Jul;8(7):e1001057.
26. Habtamu M, van den Boogaard J, Ndaro A, Buretta R, Irongo CF, Lega DA, et al. Light-emitting diode with various sputum smear preparation techniques to diagnose tuberculosis. Int J Tuberc Lung Dis. [Comparative Study
Research Support, Non-U.S. Gov't]. 2012;16(3):402-7.
27. Marais BJ, Brittle W, Painczyk K, Hesseling AC, Beyers N, Wasserman E, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clin Infect Dis. [Comparative Study
Evaluation Studies]. 2008 Jul 15;47(2):203-7.
28. Ba F, Rieder HL. A comparison of fluorescence microscopy with the Ziehl-Neelsen technique in the examination of sputum for acid-fast bacilli. Int J Tuberc Lung Dis. [Comparative Study]. 1999 Dec;3(12):1101-5.
29. Cattamanchi A, Davis JL, Worodria W, den Boon S, Yoo S, Matovu J, et al. Sensitivity and specificity of fluorescence microscopy for diagnosing pulmonary tuberculosis in a high HIV prevalence setting. Int J Tuberc Lung Dis. [Comparative Study
Evaluation Studies
Research Support, N.I.H., Extramural]. 2009 Sep;13(9):1130-6.
30. Chaidir L, Parwati I, Annisa J, Muhsinin S, Meilana I, Alisjahbana B, et al. Implementation of LED fluorescence microscopy for diagnosis of pulmonary and HIV-associated tuberculosis in a hospital setting in Indonesia. PLoS One. [Research Support, Non-U.S. Gov't]. 2013;8(4):e61727.

**Checklist for optimizing tuberculosis detection manuscript submitted to BMJ open**

| Section and Topic | Item # | | On page # |
|---|---|---|---|
| TITLE/ABSTRACT/ KEYWORDS | 1 | *Optimizing Mycobacterium tuberculosis detection in resource limited settings*: a comparison between different types of florescence smear microscopy techniques with the traditional Zeal Nielsen's (ZN) smear microscopy under routine working conditions of a resource limited setting. | 1 |
| INTRODUCTION | 2 | The diagnostic accuracies of the standard but more expensive mercury vapor lamp (FM) florescence microscope; the newer, less expensive LED based CyScope® (CY); and the Primo Star iLED (PiLED) florescence microscopes were compared to that of the conventional light ( ZN) microscope against the gold (reference) standard of liquid culture in automated BACTEC MGIT 960™ machine | 2 |
| METHODS | | | |
| *Participants* | 3 | 450 sputum samples from 150 new patients with clinical diagnosis of pulmonary tuberculosis were studied from the main tuberculosis case referral center in northern Nigeria. | 3 |
| | 4 | Participant recruitment: sputum samples from new patients with clinical diagnosis of pulmonary tuberculosis based on presenting symptoms independent of the outcome of the routine (standard of care TB test) were selected for this study. | 3 |
| | 5 | Participant sampling: Sputum samples of consecutive new but de-identified patients with clinical diagnosis of pulmonary tuberculosis were sampled. | 3 |
| | 6 | Data collection: This was a cross-sectional study where three sputum samples collected from patients over 24 hour period (spot-morning-spot) for the purpose of routine clinical care were used. | 3 |
| *Test methods* | 7 | The reference standard was sputum culture in Mycobacterium Growth Indicator Tubes (MGIT) and incubated in the automated BACTEC MGIT 960™ machine. Liquid cultures are more sensitive, fast and reliable techniques for the diagnosis of pulmonary tuberculosis | 4 |
| | 8 | The three sputum samples collected from each patient were pooled into a single specimen. Four direct smears were made from each of the pooled specimen. The first slide was stained with Ziehl Neelsen (ZN) while the second, third and fourth slides were Auramine-rhodomine stained stain and examined under FM, CY and PiLED florescence microscopes respectively. | 3 |

| | | | |
|---|---|---|---|
| | 9 | Positive smears were graded as scanty if there were: 1-9 AFBs in at least 100 fields; +1: 10-99 in 100 fields; +2: 1-10 per field in at least 50 fields; and +3: if there were more than 10 per field in at least 20 fields. Absence of any AFB was graded as negative smear.<br>Cultures that indicated positive growth were removed from the MGIT machine and the presence of AFB confirmed by ZN smear microscopy. Those that do not indicate growth after 42 days were classified as negatives as recommended by the manufacturer and as was the standard practice. | 3-4 |
| | 10 | Three experts were involved with the execution and interpretation of the smear microscopy tests while two were responsible for the reference (culture) tests. All experts hold a minimum of bachelor's degree in microbiology and had worked for at least 2 years prior to the study conduct. | 4 |
| | 11 | Experts were blinded on the clinical diagnoses and outcome of the tests done for clinical care purposes. | 4 |
| *Statistical methods* | 12 | Diagnostic accuracies were measured using decision matrix tables and matched sample tables for positive and negative final outcomes as previously recommended for comparative analysis of diagnostic procedures performed on same samples (patients) by Hawass NE in Br J Radiol. 1997 Apr;70(832):360-6. Differences in tests performances together with their 95% confidence intervals were evaluated using the McNemar's test. | 4-5 |
| | 13 | Routine laboratory quality assurance measures were used to assess tests reliability, not reported in this manuscript. | 0 |
| RESULTS | | | |
| *Participants* | 14 | This study was conducted from July 3, to September 26, of 2009 | 3 |
| | 15 | Clinical and demographic information of participants was excluded to minimize bias and ensure complete de-identification of data | 3 |
| | 16 | Main inclusion criteria were clinical diagnoses of TB, patient not on anti-TB drugs at the time of the study and provision of three sputum samples. 205 patients with clinical TB were seen over the study period. 10 were on anti-TB drugs at presentation, 16 failed to provide any sputum sample, 10 provided between 1 to 2 samples while culture results of 19 patients were contaminated and therefore all were excluded from the analyses. | 3 |
| *Test results* | 17 | There was no TB treatment administered within the 24 hour interval in which samples were collected. The index and reference tests were performed on the same sample. | 3 |

| | | | |
|---|---|---|---|
| | 18 | Disease severity was not graded in this study. However, there were patients with clinical diagnoses of pulmonary diseases other than tuberculosis. Most of those patients were not requested to provide sputum samples for tuberculosis test and such samples were not targets of this study. | 0 |
| | 19 | A cross tabulation of the results of the index tests by the results of the reference standard were provided in Tables 1 and 2. | 7-8 |
| | 20 | The tests were performed on sputum samples provided by patients, no intervention was involved and therefore no adverse events monitored. | 0 |
| *Estimates* | 21 | Details of the estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals) were provided in Table 4 of the manuscript. | 10 |
| | 22 | The outcomes of the index and reference standard test were binary (yes, or no). There were no outliers, no missing data. | 0 |
| | 23 | Variability of diagnostic accuracy between the smear microscopy readers was not evaluated, a smear was positive if AFBs were seen by all the three readers. | 4 |
| | 24 | Estimates of test reproducibility was not evaluated | 0 |
| DISCUSSION | 25 | The high sensitivity for AFB detection shown by the inexpensive light-emitting diode based Primo Star iLED florescence microscope in the hard operational conditions of a resource limited setting underscores the potentials for its scalability to remote clinics with similar operational conditions. This will increase TB detection and improve TB treatment uptakes in high burden settings with scarce resources. The optimal exposure duration for decontamination of sputum samples prior to examination and culture using the n-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) reagent in this setting is 15 minutes. | 13 |

# Optimizing Mycobacterium tuberculosis detection in resource limited settings

| | |
|---|---|
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# Optimizing Mycobacterium tuberculosis detection in resource limited settings

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## Abstract

**Objectives**: The light-emitting diode (LED) fluorescence microscopy has made acid-fast bacilli (AFB) detection faster and efficient although its optimal performance in resource limited settings is still being studied. We assessed the optimal performances of light and fluorescence microscopy in routine conditions of a resource limited setting and evaluated the digestion time for sputum samples for maximum yield of positive cultures.

**Design**: Cross-sectional study

Setting: Facility-based involving samples of routine patients receiving tuberculosis treatment and care from the main tuberculosis case referral center in northern Nigeria.

Participants: The study included 450 sputum samples from 150 new patients with clinical diagnosis of pulmonary tuberculosis.

**Methods**: The 450 samples were pooled into 150 specimens, examined independently with mercury vapor lamp (FM), LED CysCope® (CY) and Primo Star iLED (PiLED) fluorescence microscopies, and with the Ziehl Neelsen (ZN) microscopy to assess the performance of each technique compared to liquid culture. The cultured specimens were decontaminated with BD Mycoprep (4%NaOH-1%NLAC and 2.9% sodium citrate) for 10, 15 and 20 minutes before incubation in Mycobacterium Growth Incubator Tube (MGIT) system and growth examined for acid-fast bacilli (AFB)

**Results**: Of the 150 specimens examined by direct microscopy: 44 (29%), 60 (40%), 49 (33%) and 64 (43%) were AFB positive by ZN, FM, CY, and iLED microscopy respectively. Digestion of sputum samples for 10, 15 and 20 minutes yielded mycobacterial growth in 72 (48%), 81 (54%) and 68 (45%) of the digested samples respectively after incubation in the MGIT system.

**Conclusions**: In routine laboratory conditions of a resource-limited setting, our study has demonstrated the superiority of fluorescence microscopy over the conventional ZN technique. Digestion of sputum samples for 15minutes yielded more positive cultures


**Keywords**: Tuberculosis, Microbiology, Respiratory infections

**Word count**: 3,399

**Article focus**:

1. What is the optimal performance of the new light-emitting diode (LED) florescence microscopy in routine conditions of a resource limited setting and how does it compare to the traditional light microscopy using Ziehl Neelsen (ZN) technique for AFB smear microscopy?
2. What is the optimal digestion time for sputum samples for maximum yield of positive cultures in routine conditions of a resource limited setting?
3. Despite the operational challenges of resource limited settings flourescence microscopy has better optimal performance compared to the traditional light microscopy using Ziehl Neelsen (ZN) technique for AFB smear microscopy.

**Significance**:

1. Most of the settings with highest burden of Tuberculosis in the world are limited in resource needed to effectively detect and manage cases of the disease.
2. The WHO has recommended the phasing out of the simple, inexpensive but less sensitive ZN technique with the more sensitive florescence technique for better TB detection in all settings.
3. Evaluating the performance of the more sensitive florescence microscopy against the less sensitive ZN microscopy in unaltered working conditions of resource limited settings is relevant for effective TB control.

**Strength and Limitations**:

1. The design of this study allows test performance assessment under routine (real) resource constrain settings.
2. Blinding the microscopist on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of direct smear results
3. However, our inability to obtain participant HIV status deprived us the ability to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures.

**Introduction**

Globally, an estimated 1.4 million death occurred in 2011 as a result of infection with tuberculosis, one-fourth of the death was associated with HIV infection and most of it in resource limited settings (1) where the burden of HIV infection is high. In Africa, TB is the leading cause of death among HIV infected persons as the continent harbors eighty percent of the world HIV-TB cases (2). Nigeria and the Republic of South Africa are among the top five TB burden nations of the world. Sensitive, specific, and inexpensive point-of-care diagnostic tools for rapid TB detection are lacking posing challenges to the optimal diagnosis of TB in resource limited settings (RLS), particularly among persons with HIV infections whose sputum specimen often lack sufficient quantity of acid-fast bacilli (AFB) detectable on routine smear examination. (3, 4)

The simplicity, inexpensiveness and predictive power of the Ziehl Neelsen sputum smear microscopy make it the applicable laboratory diagnostic tool of choice for tuberculosis in resource limited settings. (5) A properly done positive sputum-smear is highly predictive of active TB (6, 7). Unfortunately, the sensitivity of sputum-smear microscopy is low and its performance is often affected by lack of proper maintenance of equipment; trained manpower; and quality assurance system in settings where resources are scarce. (8) Even in centers with good quality assurance measures, smear microscopy detects only 40-50% of TB cases compared to about 80% yield by culture (9). However, it remains the only available tool for bacteriologic diagnosis of TB in most RLS. Culture techniques are highly sensitive and specific, but the cost, technical complexity and time delay before results are available make culture not scalable for rapid detection and treatment of tuberculosis. Recent advances have made it possible to use molecular technology to detect Mycobacterial deoxyribonucleic acid (DNA) with great accuracy. However these technologies still require a fairly developed laboratory with a reliable source of electricity.

Detection of smear positive cases is the priority in TB control programs. These cases are infectious and contribute substantially to the transmission of the disease. (10, 11) In recent years, several new laboratory techniques have been developed to significantly improve the ability to diagnose active TB in populations. Fluorescence microscopy improves the sensitivity of sputum-smear detection of TB and has good specificity relative to the conventional ZN method. Inexpensive light-emitting diode-based (LED) fluorescence

microscopes have been developed to make microscopy of smears stained by Auramine fluorescence dye easier, cheaper and faster with potentials for scalability to remote clinics in RLS. (5, 12-14).

This study compared the performance of the standard but more expensive mercury vapor lamp (FM) fluorescence microscope; the newer, less expensive LED based CysCope® (CY) (Partec, Görlitz, Germany); and the Primo Star iLED (PiLED) (Carl Zeiss Inc. Germany) fluorescence microscopes in comparison to the conventional light ( ZN) microscope against the gold (reference) standard of liquid culture in automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems) for the detection of AFB from sputum samples of patients with clinical pulmonary tuberculosis (TB). In addition, we evaluated the optimal digestion time with *Mycoprep (4%NaOH-1%NLAC and 2.9% sodium citrate)* for liquid culture in this population. Prolonged decontamination time is reported to reduce the number of viable bacilli and different digestion methods yield variable number of positive cultures (15-17).

## Methods

*Study population*

Three sputum samples (spot-morning-spot) were collected from 150 TB treatment naïve, consecutive patients with clinical diagnosis of pulmonary tuberculosis at the National TB and Leprosy Training Center (NTBLTC), Zaria, Nigeria. Routine and referred cases of TB from northern Nigerian region receive TB treatment and care at this facility. The spot-morning-spot sample collection was the standard routine for the diagnosis of TB in Nigeria at the time of the study. The flow chart in **Figure 1** provides an overview of the study design. Ethical review was waived because in the opinion of the study center review committee there was no potential risk to participants' safety, privacy or confidentiality since there was no formal contact between investigators and participants either directly (interview, questionnaires, etc.) or indirectly (medical records, personal identifiers etc.). The sputum specimens provided for routine clinical care services were completely anonymized before they were analyzed for the study and there was no risk that the pooled samples can be de-anonymized through data linkages. This study was conducted from July to September of 2009.

**Figure 1**. Flow chart for the study design involving 450 sputum samples from 150 patients with clinical TB

*Smear Microscopy*

The three sputum samples from each patient were pooled. Four direct smears were made from each of the pooled sputum samples with applicator sticks on clean grease-free slides; measuring 2 cm x 3 cm, and not too thick. The first slide was stained with Ziehl Neelsen (ZN) stain according to the World Health Organization (WHO) recommended protocol described elsewhere. (18) Briefly, the smear was air dried and fixed by gently passing it over a flame 2-3 times. One percent strong carbol-fuchsin was applied to the slide and heated with a Bunsen flame intermittently 3 times and allowed to stain for 15 minutes. The stain was rinsed off with tap water and 3% acid alcohol was applied on the smear for 3 minutes. Methylene blue (0.3%) was then added for up to 1 minute and rinsed off with tap water. Positive and negative controls were included in the process. Slide examination was done with light microscope at 1,000x magnification and the AFBs identified were graded according to the International Union against Tuberculosis and Lung Disease (IUATLD) and the WHO smear grading scale. (19)

The second, third and fourth slides were air-dried, heat-fixed and stained with Auramine O-phenol for 15 minutes rinsed with tap water, decolorized with 1 percent acid alcohol for 2 minutes then rinsed with water and counterstained with 0.1 percent potassium permanganate for 2 minutes. The slides were rinsed with water then allowed to dry in air before examination under FM, CY and PiLED microscopes respectively. The AFBs were visualized and graded at magnifications of 200× and 400× according to a protocol describe by Lehman (20). A total of 100 microscopic fields were examined before a slide is recorded as positive (if at least an AFB was seen) or negative (if no AFB was seen). Positive smears were graded as scanty (actual) if there were: 1-9 AFBs in at least 100 fields; +1: 10-99 in 100 fields; +2: 1-10 per field in at least 50 fields; and +3: if there were more than 10 per field in at least 20 fields. Absence of any AFB was graded as negative smear. Each slide was examined by three independent readers to ascertain the presence of AFB and grade positive smears. The slide readers were blinded on the clinical and laboratory diagnoses of the subjects whose samples were studied.

*Specimen culture*

Each of the 150 pooled sputum specimens were decontaminated with BD Mycoprep$^{TM}$ (Beckton Dickinson Diagnostic Systems, Sparks, Maryland, USA) which consists of 1% N-acetyl-L-cysteine (NALC), 4% sodium hydroxide and 2.9% sodium citrate. An equal amount of Mycoprep was added to the sputum, homogenized and

allowed to act for 10, 15 and 20 minutes respectively. After the respective digestion period, phosphate buffered saline was added to stop the digestion and decontamination reaction. This was then centrifuged under refrigerated conditions with uniform centrifugation time of 15 minutes and a Relative Centrifugal force (RCF) of 3000 x g. The supernatants were discarded and the sediments re-suspended with 1 ml of phosphate buffered saline to make a final volume of 2 ml. Then 0.5 ml of each was inoculated into Mycobacterium Growth Indicator Tubes (MGIT) and incubated in the automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems), which monitors growth. Positive and negative control culture tubes were also set up along each test using *Mycobacterium tuberculosis* H37Ra (ATCC 25177 – attenuated strain) and sterile distilled water respectively.

The cultures that indicated positive growth were removed from the machine and the presence of AFB confirmed by ZN smear microscopy. Those that do not indicate growth after 42 days were classified as negatives as recommended by the manufacturer and as was the standard practice. All cultures were done at Zankli Research laboratory while all microscopy were performed at the NTBLTC, Zaria to minimize interference with the outcome of culture or direct microscopy results.

*Statistical analysis*

The raw data generated from the smear microscopic examinations of the 150 matched samples by the four different microscopes as well as the data generated after same samples were cultured following decontaminations (digestions) at 10, 15 and 20 minutes were organized into cell counts in which descriptive values were provided along with frequencies and proportions. Measures of accuracy for the different smear examinations: sensitivity, specificity, positive and negative predictive values were evaluated through decision matrix tables while concordance and disagreements between the microscopic examination by each of the three fluorescence microscopes and the conventional light (ZN) microscope based on the final classification of samples by the gold standards (outcomes MGIT cultures) were examined in matched sample tables for positive and negative final outcomes as previously recommended for comparative analysis of diagnostic procedures performed on same samples (patients) (21). Comparison between the fluorescence microscopies to the conventional ZN microscopy for differences in performance together with their 95% confidence intervals was done using the McNemar's test. Significance of association was set at 0.05 alpha and all probabilities were two tailed.

**Results**

Of the 150 matched (paired) samples, AFBs were detected in: 44 (29%) of the ZN smears examined under light microscope; 60 (40%) of the AR smears examined with FM; 49 (33%) of the AR smears examined with CY; and 64 (44%) AR smears examined with PiLED fluorescence microscopes. MGIT cultures of the 150 specimen yielded 72 (48%), 81 (54%) and 68 (45%) AFB confirmed growths after decontamination for 10 (A), 15 (B) and 20 (C) minutes respectively prior to incubation.).

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Among the positive AFB tests graded, ZN light microscopy produced the highest proportion of samples with scanty AFBs on examination 21/44 (48%), followed by PiLED fluorescence microscopy 12/64 (19%), then CY 8/49 (16%) and FM had the least scanty AFB positive counts 3/60 (5%). However, after the samples with scanty positive AFBs where incubated in A, B and C MGIT culture groups, the proportions of confirmed myco-bacterial growths in the sequence above were as follows: (i) ZN: 14/21 (67%), 9/21 (42%) and 11/21 (52%); (ii) PiLED: 5/12 (42%), 7/12 (58%) and 5/12 (42%); (iii) CY: 6/8 (75%), 4/8 (50%) and 3/8 (38%); lastly (iv) FM: 0/3 (0%), 1/3 (33%) and 1/3 (33%). A summary of the positive AFB outcomes from the direct smear examina-tions by microcopy types, grades and the proportions of graded smears confirmed by the MGIT cultures were provided in **Table 1**.

**Table 1**. Proportions of positive graded direct smears confirmed by cultured specimen decontaminated at 10 (A), 15 (B) and 20(C) minutes obtained from 150 matched samples

| Direct smear (DS) | Grade | Frequency | MGIT cultured specimen (Gold standards) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A | | B | | C | |
| | | | n | % | n | % | n | % |
| ZN | scanty | 21 | 14 | 66.7 | 9 | 42.1 | 11 | 52.4 |
| | +1 | 13 | 12 | 92.3 | 11 | 84.6 | 10 | 76.9 |
| | +2 | 5 | 5 | 100.0 | 4 | 80.0 | 5 | 100.0 |
| | +3 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| FM | Scanty | 3 | 0 | 0.0 | 1 | 33.3 | 1 | 33.3 |
| | +1 | 34 | 24 | 70.6 | 19 | 55.9 | 17 | 50.0 |
| | +2 | 18 | 16 | 88.9 | 12 | 66.7 | 13 | 72.2 |
| | +3 | 5 | 4 | 80.0 | 4 | 80.0 | 4 | 80.0 |
| CY | Scanty | 8 | 6 | 75.0 | 4 | 50.0 | 3 | 37.5 |
| | +1 | 30 | 22 | 73.3 | 18 | 60.0 | 19 | 63.3 |
| | +2 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| | +3 | 6 | 6 | 100.0 | 6 | 100.0 | 6 | 100.0 |
| PiLED | Scanty | 12 | 5 | 41.7 | 7 | 58.3 | 5 | 41.7 |
| | +1 | 36 | 22 | 61.1 | 20 | 55.6 | 18 | 50.0 |
| | +2 | 13 | 13 | 100.0 | 10 | 76.9 | 13 | 100.0 |
| | +3 | 3 | 3 | 100.0 | 2 | 66.7 | 3 | 100.0 |

ZN: Ziehl Neelsen microscopy using the conventional light microscope
FM: Fluorescence microscopy with mercury vapor lamp microscope
CY: Fluorescence microscopy with CysCope light emitting diode microscope
PiLED: Fluorescence microscopy with Primo Star iLED light emitting diode microscope

A comparison of the direct smear tests results with the final culture outcomes to measure the performances of the direct smear techniques against the gold standards showed that of the 44 AFB positive samples identified by the ZN microscopy; 36 (81.8%), 29 (65.9%) and 31 (70.5%) were culture confirmed after 10, 15 and 20 minutes decontamination respectively. Similarly, of the 60, 49 and 64 AFB positive samples identified by the FM, CY and PiLED microscopy techniques the frequencies and proportions of the culture confirmed samples were: 44 (73.3%) 36 (60.0%) and 35 (58.3%) for FM; 39 (79.6%), 33 (67.3%) and 33 (67.3%) for CY;

and 43 (67.2%) 39 (56.3%) and 39 (56.3%) for PiLED at decontamination times of 10, 15 and 20 minutes respectively. There were near identical frequency distributions for the true positive (TP), false positive (FP), false negative (FN) and true negative (TN) for the ZN and CY microscopy techniques while the FM microscopy yielded a pattern of distribution similar to that of PiLED technique as shown in the decision matrix of **Table 2**.

**Table 2**. Decision matrix for the evaluation of performance of the direct smear methods against the different gold standards for the 150 matched samples

| Direct Smear (DS) | DS Outcome | Gold standards: MGIT cultures | | | | | |
|---|---|---|---|---|---|---|---|
| | | A : culture positive =72 | | B: culture positive =81 | | C: culture positive = 68 | |
| | | P | N | P | N | P | N |
| ZN | P | 36 | 08 | 29 | 15 | 31 | 13 |
| DS+ = 44 | N | 36 | 70 | 52 | 54 | 37 | 69 |
| FM | P | 44 | 16 | 36 | 24 | 35 | 25 |
| DS+ =60 | N | 28 | 62 | 45 | 45 | 33 | 57 |
| CY | P | 39 | 10 | 33 | 16 | 33 | 16 |
| DS+ =49 | N | 33 | 68 | 48 | 53 | 35 | 66 |
| PiLED | P | 43 | 21 | 39 | 25 | 39 | 25 |
| DS+ =64 | N | 29 | 57 | 42 | 44 | 29 | 57 |

Culture specimen decontaminated by NaOH-NALC : A for 10 minutes; B for 15minutes; C for 20 minutes
DS+ = number of samples (out of 150 samples) that were AFB positive by direct smear
P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor fluorescence microscopy; CY=CysCope fluorescence microscopy and PiLED= Primo Star iLED fluorescence microscopy

The levels of agreement and differences between the convention ZN microscopy and each of the fluorescence microscopy techniques with respect to the final culture results were demonstrated by the concordance and discordance cells displayed in **Table 3**. Cells were discordant if the outcomes of the two techniques disagreed otherwise they were concordant. The statistical significance of the difference in the performance of the techniques compared was derived from the discordant cell. The sum of the frequencies of the discordant cells for example that explained the differences between ZN and CY were 7, 4 and 2 for the positive culture results which showed less consistent divergence compared to 6, 6, and 7 for the negative culture results of the same

techniques. The sum of the discordant frequencies between ZN and FM, and between ZN and PiLED were higher relative to those between ZN and CY.

**Table 3**: Matched sample frequency cells for the agreement and discordance between the standard of care (ZN smear) and FM, CY and PiLED smears on the culture positive and negative specimens

| | | | FM | | CY | | PiLED | |
|---|---|---|---|---|---|---|---|---|
| Culture Positive | | | P | N | P | N | P | N |
| A: n= 72 | ZN | P | 35 | 1 | 34 | 2 | 35 | 1 |
| | | N | 9 | 27 | 05 | 31 | 08 | 28 |
| B: n= 81 | ZN | P | 29 | 0 | 29 | 0 | 29 | 0 |
| | | N | 7 | 45 | 4 | 48 | 10 | 42 |
| C: n= 68 | ZN | P | 29 | 2 | 31 | 0 | 29 | 2 |
| | | N | 6 | 31 | 2 | 35 | 10 | 27 |
| Culture Negative | | | | | | | | |
| A: n= 78 | ZN | P | 6 | 2 | 7 | 2 | 9 | 0 |
| | | N | 7 | 63 | 4 | 65 | 12 | 57 |
| B: n= 69 | ZN | P | 11 | 1 | 6 | 2 | 14 | 0 |
| | | N | 10 | 47 | 4 | 57 | 10 | 45 |
| C: n= 82 | ZN | P | 11 | 1 | 10 | 1 | 13 | 0 |
| | | N | 12 | 58 | 6 | 65 | 10 | 59 |

P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor fluorescence microscopy; CY=CysCope fluorescence microscopy and PiLED= Primo Star iLED fluorescence microscopy
A: cultured samples decontaminated for 10 minutes; B: cultured samples decontaminated for 15 minutes; C: cultured samples decontaminated for 20 minutes

The ZN technique was consistently less sensitive compared to any of the fluorescence techniques against any of the gold standards. The FM fluorescence technique was: 11.1%, 8.6% and 5.9%; CY method was: 4.2%, 4.9% and 3.1% while PiLED was 9.7%, 12.3%, and 11.8% more sensitive than ZN microscopy for the different digestion times of 10, 15 and 20 minutes respectively ( $P_{fm}$= 0.027, 0.023, 0.289; $P_{cy}$ = 0.450, 0.134, 0.479 and $P_{iled}$ =0.046, 0.004, 0.043). Conversely, the FM was: 10.2%, 13.1% and 14.6%; CY was: 2.5% 2.5% and 3.6%; and PiLED was: 16.6%, 1.4% and 14.6% less specific compared to the ZN microscopy for the same digestion times above (*Pfm*= 0.182, 0.016, 0.006; $P_{cy}$ = 0.684, 0.684, 0.131 and $P_{iled}$ =0.006, 0.131, and 0.004). They were also less predictive than the ZN technique (**Table 4**).

**Table 4**. Summary measures of performance of the direct smear diagnostic tests for 150 matched samples and the statistical differences in sensitivity and specificity of FM, CY and PiLED in comparison to ZN

| Gold Stand | Direct Smear | Sn | Sp | PPV | NPV | SnD [95% CI] | p-value* | SpD [95% CI] | p-value* |
|---|---|---|---|---|---|---|---|---|---|
| A | ZN | 50.0 | 89.7 | 81.8 | 66.0 | | | | |
| | FM | 61.1 | 79.5 | 73.3 | 68.9 | 11.1 [3.0, 19.0] | 0.027 | 10.2 [2.5, 17.4] | 0.182 |
| | CY | 54.2 | 87.2 | 79.6 | 67.3 | 4.2 [-2.9, 11.3] | 0.450 | 2.5 [-3.6, 8.6] | 0.684 |
| | PiLED | 59.7 | 73.1 | 67.2 | 66.3 | 9.7 [1.9, 17.5] | 0.046 | 16.6 [8.6, 24.6] | 0.001 |
| B | ZN | 35.8 | 78.3 | 65.9 | 50.9 | | | | |
| | FM | 44.4 | 65.2 | 60.0 | 50.0 | 8.6 [2.4, 14.8] | 0.023 | 13.1 [3.9, 22.1] | 0.016 |
| | CY | 40.7 | 76.8 | 67.3 | 52.5 | 4.9 [0.2, 9.6] | 0.134 | 2.5 [-4.4, 9.4] | 0.684 |
| | PiLED | 48.1 | 63.8 | 60.9 | 51.2 | 12.3 [4.9, 19.6] | 0.004 | 1.4 [-5.5, 7.9] | 0.683 |
| C | ZN | 45.6 | 84.1 | 70.5 | 65.1 | | | | |
| | FM | 51.5 | 69.5 | 58.3 | 63.3 | 5.9 [-2.1, 13.9] | 0.289 | 14.6 [6.6, 22.6] | 0.006 |
| | CY | 48.5 | 80.5 | 67.3 | 65.3 | 3.1 [-0.9, 7.1] | 0.479 | 3.6 [-2.6, 9.8] | 0.131 |
| | PiLED | 57.4 | 69.5 | 60.9 | 66.3 | 11.8 [2.2, 21.4] | 0.043 | 14.6 [7.5, 21.7] | 0.004 |

Sn: Sensitivity; Sp: Specificity; PPV: positive predictive value; NPV; Negative predictive value
SnD: Sensitivity difference; percentage points above ZN performance
SpD: Specificity difference; percentage points below ZN performance
CI: Confidence Interval; *: Chi-square p-value generated from McNemar's test

## Discussion

The findings of this study further confirm the previously reported superior performance of fluorescence microscopy over the conventional ZN technique for AFB detection (22-24). The direct smear staining sensitivities of FM and PiLED fluorescence techniques are on the average 10% more sensitive than the standard of care (ZN method). The reverse is however true for specificity, the fluorescence methods are less specific than ZN by similar percentage points and these differences are statistically significant. Similar trends of high sensitivity and low specificity were observed in the performance of fluorescence microscopy in comparison to the conventional Ziehl Neelsen technique (24-26) Within the fluorescence techniques FM and PiLED have similar performance measures with little or no differences which is in agreement with the reported findings by Marais et al.(27) The CY technique appears to be less sensitive compared to FM and PiLED but on the average have 4% staining sensitivity advantage over the conventional ZN. Similar findings were reported from a resource constrained setting with comparable optimal technical conditions in which such differences were found to be marginally significant statistically. (20). However, both CY and ZN staining techniques have higher specificities compared to FM and PiLED across all the three reference (gold) standards used.

Although less sensitive, the ZN staining seems to be the most predictive: a positive test has the highest chance of being a true case of TB while a negative test is less likely to harbor the disease. The CY has similar predictive probabilities; however, these measures are affected by disease burden. In high prevalence settings, the positive predictive value of a test increases because it is more likely that those who test positive truly have disease than if the test was performed in settings with low prevalence. Likewise, in settings with low prevalence it is more likely that those who test negative truly do not have the disease. In contrast, sensitivity and specificity measures are less susceptible to influence by disease prevalence. The search for an optimal tuberculosis diagnostic test is motivated by the unacceptably low sensitivity of the conventional ZN smear microscopy over which fluorescence microscopy has shown consistent superiority. In this setting, the TB prevalence is high and may be driven by HIV. Priority should therefore be given to sensitivity over predictive value since failure to detect TB in patients co-infected with HIV is more likely to increase morbidity and mortality among the HIV infected persons than false detection of TB.

In addition to improved TB case detection in samples with low-density bacilli, which is aided by its low-power objective, previous studies reported that fluorescence microscopy requires less than 25% of the time taken to read slides using the conventional ZN technique-meaning a microscopist can examine 4 times the number of slides per day with fluorescence technique.(20, 28) However, the use of mercury vapor or halogen lamp as the source of light in the standard fluorescence microscopy technique (FM) is expensive and requires frequent replacement because of their shorter life span making it economically inefficient for use in resource constrained settings. The LED based microscopes (CY and PiLED) on the other hand are structurally built to overcome the hard operational environment of RLS where continuous supply of electricity, sufficient equipment and trained personnel are a frequent challenge. The LED microscopes use lamps that are inexpensive with much longer life span. They are also simple to operate; and no dark room required.

The scanty-positive findings are more commonly seen with ZN technique and least with the FM. This could be attributed to the low-power objective of the fluorescence techniques that allow the field to be seen larger than in the conventional ZN light microscopy. Some of the samples studied may have come from HIV co-infected patients with low-density bacilli yielding scanty AFBs on the conventional ZN microscopy earning a grade of 1+ or more when viewed under the fluorescence techniques. However, the fluorescence techniques recorded the highest numbers of false positives compared to the ZN judging from the reported frequencies in the decision matrix table. This is expected because the low-threshold of the fluorescence techniques derived from their low-power objective could allow naturally fluorescence particles present in the sputum, certain spores, fungi to appear AFB positive which may otherwise be negative on ZN microscopy.  These and the fact that significant proportion of the samples studied may have come from HIV co-infected patients could also explain the particularly low specificity of the PiLED technique. Associated HIV infection was believed to account for low specificity of LED-FM microscopy compared to the conventional ZN microscopy in two previous studies in settings with high burden of the disease (29, 30)

As expected, the digestion and decontamination with n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) produces the highest yield of AFB in the sputum exposed to the reagent for 15 minutes. This shows that the manufacturer's recommended exposure time of 15 minutes works well in the hard operational environment of a resource limited setting. The 10 minutes alternative yielded a little less AFB than the 15 minutes duration probably because the time is not enough to allow complete digestion of the thick sputum which allows the release of mycobacteria and their subsequent concentration by centrifugation. The 20 minutes exposure on the other hand might have been prolonged beyond the optimal duration necessary and the toxic effect of the reagent kills not only the contaminating normal flora that may overgrow in cultures making it hard to detect the presence of the mycobacteria but also the mycobacteria itself. The success of this process is dependent on the concentration of the reagent, the exposure time, centrifugation and the temperature selected. In this case all other factors were kept constant and only the exposure time was altered.

Some of the reasons for the unusually high proportion of AFB positive, culture negative specimen could possibly be that some of the patients contrary to their claim have actually been on TB treatment at presentation. This may have adversely hindered the bacilli ability to grow, or more importantly in this case, the bacilli may have been killed by the excessive decontamination in the samples decontaminated for up to 20 minutes especially among cases with paucibacillary disease due to HIV co-infection. These patients may likely test positive to both smear microscopy and culture in repeat examinations with optimal decontamination time. Fungal infections are also not uncommon in the study area and together with some artefacts may have added to the high frequency of the smear positive, culture negative findings.

The design of this study allows test performance assessment under routine (real) resource constrain settings. The point estimates obtained are more likely to represent the expected outcomes in similar settings with high prevalence of TB. Blinding the readers on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of the direct smear results. However, our study had a few limitations: We did not obtain participants' HIV status and therefore unable to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures. The time taken to read slides was also not reflected in the analysis because only estimates of the time taken to read the slides were recorded since the different technicians that read the slides had different proficiency levels and some accurately read the slides much faster than others.

In summary, the high sensitivity for AFB detection shown by the inexpensive light-emitting diode based Primo Star iLED fluorescence microscope in the hard operational conditions of a resource limited setting underscores the potentials for its scalability to remote clinics in similar settings.. This will increase TB detection and improve TB treatment uptakes in high burden settings with scarce resources. The optimal exposure duration for decontamination of sputum samples prior to examination and culture using the n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) reagent in this setting is 15 minutes.

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**Contributorship Statement:** Contributors: AA Conceived and designed the study, data collection, revised the article's intellectual content and approved the final version for publication. She is guarantor for the intellectual content of this paper. LL designed tools for data collection, monitored data collection, revised and approved the draft paper. AN collected, cleaned, analyzed and wrote the initial draft of the paper. GA designed the analysis plan, cleaned, analyzed the data, draft and revised the paper. OO, PM, TJ, IM, EN and OnO, implemented the study, collected and cleaned the data and wrote the method section of the paper. DP contributed in the conception and design of data collection, supervised data collection and revised the draft paper.

**Data Sharing Statement:** There are no additional unpublished data from this study. The current data de-identified data are with the PI (AA). Data will remain de-identified and will be shared as such should the need arises.

**Competing interest**: All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf and declare: no support from any organisation for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

## References

1. De Cock KM, Simone PM, Davison V, et al. The new global health. Emerg Infect Dis. 2013 Aug;19(8):1192-7.
2. Corbett EL, Watt CJ, Walker N, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. [Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.
Review]. 2003 May 12;163(9):1009-21.
3. Young DB, Perkins MD, Duncan K, et al. Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest. [Research Support, Non-U.S. Gov't
Review]. 2008 Apr;118(4):1255-65.
4. Getahun H, Harrington M, O'Brien R, et al. Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. Lancet. [Review]. 2007 Jun 16;369(9578):2042-9.
5. Somoskovi A, Hotaling JE, Fitzgerald M, et al. Lessons from a proficiency testing event for acid-fast microscopy. Chest. [Research Support, U.S. Gov't, P.H.S.]. 2001 Jul;120(1):250-7.
6. Van Deun A, Salim AH, Cooreman E, et al. Optimal tuberculosis case detection by direct sputum smear microscopy: how much better is more? Int J Tuberc Lung Dis. [Evaluation Studies]. 2002 Mar;6(3):222-30.
7. Cole EC, Cook CE. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. Am J Infect Control. [Review]. 1998 Aug;26(4):453-64.
8. Parsons LM, Somoskovi A, Gutierrez C, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin Microbiol Rev. [Review]. 2011 Apr;24(2):314-50.
9. Monkongdee P, McCarthy KD, Cain KP, et al. Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. Am J Respir Crit Care Med. [Evaluation Studies
Research Support, U.S. Gov't, Non-P.H.S.]. 2009 Nov 1;180(9):903-8.
10. Cheng S, Chen W, Yang Y, et al. Effect of Diagnostic and Treatment Delay on the Risk of Tuberculosis Transmission in Shenzhen, China: An Observational Cohort Study, 1993-2010. PLoS One. 2013;8(6):e67516.
11. Tadesse T, Demissie M, Berhane Y, et al. Incidence of smear-positive tuberculosis in Dabat, northern Ethiopia. Int J Tuberc Lung Dis. [Research Support, U.S. Gov't, Non-P.H.S.]. 2013 May;17(5):630-5.
12. Steingart KR, Henry M, Ng V, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. [Comparative Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.
Review]. 2006 Sep;6(9):570-81.
13. Hooja S, Pal N, Malhotra B, et al. Comparison of Ziehl Neelsen & Auramine O staining methods on direct and concentrated smears in clinical specimens. Indian J Tuberc. [Comparative Study]. 2011 Apr;58(2):72-6.
14. Prasanthi K, Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. Indian J Med Microbiol. [Comparative Study]. 2005 Jul;23(3):179-81.
15. Abe C. [Standardization of laboratory tests for tuberculosis and their proficiency testing]. Kekkaku. [Review]. 2003 Aug;78(8):541-51.

16. Allen BW. Comparison of three methods for decontamination of faeces for isolation of Mycobacterium tuberculosis. Tubercle. [Comparative Study]. 1991 Sep;72(3):214-7.
17. Krasnow I, Wayne LG. Comparison of methods for tuberculosis bacteriology. Appl Microbiol. [Comparative Study]. 1969 Nov;18(5):915-7.
18. Selvakumar N, Rahman F, Rajasekaran S, et al. Inefficiency of 0.3% carbol fuchsin in ziehl-neelsen staining for detecting acid-fast bacilli. J Clin Microbiol. [Comparative Study
Evaluation Studies
Research Support, Non-U.S. Gov't]. 2002 Aug;40(8):3041-3.
19. http://www.uphs.upenn.edu/bugdrug/antibiotic_manual/IUATLD_afb%20microscopy_guide.pdf. Accessed last on December 25.
20. Lehman LG, Ngapmen Yamadji AL, Ngo Sack F, et al. The CyScope(R) fluorescence microscope, a reliable tool for tuberculosis diagnosis in resource-limited settings. Am J Trop Med Hyg. [Research Support, Non-U.S. Gov't]. 2010 Oct;83(4):906-8.
21. Hawass NE. Comparing the sensitivities and specificities of two diagnostic procedures performed on the same group of patients. Br J Radiol. [Comparative Study]. 1997 Apr;70(832):360-6.
22. Hung NV, Sy DN, Anthony RM, et al. Fluorescence microscopy for tuberculosis diagnosis. Lancet Infect Dis. [Comment
Letter]. 2007 Apr;7(4):238-9; author reply 9-40.
23. Van Deun A, Chonde TM, Gumusboga M, et al. Performance and acceptability of the FluoLED Easy module for tuberculosis fluorescence microscopy. Int J Tuberc Lung Dis. [Comparative Study
Evaluation Studies
Research Support, U.S. Gov't, Non-P.H.S.]. 2008 Sep;12(9):1009-14.
24. Ulukanligil M, Aslan G, Tasci S. A comparative study on the different staining methods and number of specimens for the detection of acid fast bacilli. Mem Inst Oswaldo Cruz. [Comparative Study]. 2000 Nov-Dec;95(6):855-8.
25. Cuevas LE, Al-Sonboli N, Lawson L, et al. LED fluorescence microscopy for the diagnosis of pulmonary tuberculosis: a multi-country cross-sectional evaluation. PLoS Med. [Controlled Clinical Trial
Multicenter Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.]. 2011 Jul;8(7):e1001057.
26. Habtamu M, van den Boogaard J, Ndaro A, et al. Light-emitting diode with various sputum smear preparation techniques to diagnose tuberculosis. Int J Tuberc Lung Dis. [Comparative Study
Research Support, Non-U.S. Gov't]. 2012;16(3):402-7.
27. Marais BJ, Brittle W, Painczyk K, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clin Infect Dis. [Comparative Study
Evaluation Studies]. 2008 Jul 15;47(2):203-7.
28. Ba F, Rieder HL. A comparison of fluorescence microscopy with the Ziehl-Neelsen technique in the examination of sputum for acid-fast bacilli. Int J Tuberc Lung Dis. [Comparative Study]. 1999 Dec;3(12):1101-5.
29. Cattamanchi A, Davis JL, Worodria W, et al. Sensitivity and specificity of fluorescence microscopy for diagnosing pulmonary tuberculosis in a high HIV prevalence setting. Int J Tuberc Lung Dis. [Comparative Study
Evaluation Studies
Research Support, N.I.H., Extramural]. 2009 Sep;13(9):1130-6.

30. Chaidir L, Parwati I, Annisa J, et al. Implementation of LED fluorescence microscopy for diagnosis of pulmonary and HIV-associated tuberculosis in a hospital setting in Indonesia. PLoS One. [Research Support, Non-U.S. Gov't]. 2013;8(4):e61727.

**Optimizing Mycobacterium tuberculosis detection in resource limited settings**

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## Abstract

**Objectives**: The light-emitting diode (LED) fluorescence microscopy has made acid-fast bacilli (AFB) detection faster and efficient although its optimal performance in resource limited settings is still being studied. We assessed the optimal performances of light and fluorescence microscopy in routine conditions of a resource limited setting and evaluated the digestion time for sputum samples for maximum yield of positive cultures.

**Design**: Cross-sectional study

Setting: Facility-based involving samples of routine patients receiving tuberculosis treatment and care from the main tuberculosis case referral center in northern Nigeria.

Participants: The study included 450 sputum samples from 150 new patients with clinical diagnosis of pulmonary tuberculosis.

**Methods**: The 450 samples were pooled into 150 specimens, examined independently with mercury vapor lamp (FM), LED CysCope® (CY) and Primo Star iLED (PiLED) fluorescence microscopies, and with the Ziehl Neelsen (ZN) microscopy to assess the performance of each technique compared to liquid culture. The cultured specimens were decontaminated with BD Mycoprep (4%NaOH-1%NLAC and 2.9% sodium citrate) for 10, 15 and 20 minutes before incubation in Mycobacterium Growth Incubator Tube (MGIT) system and growth examined for acid-fast bacilli (AFB)

**Results**: Of the 150 specimens examined by direct microscopy: 44 (29%), 60 (40%), 49 (33%) and 64 (43%) were AFB positive by ZN, FM, CY, and iLED microscopy respectively. Digestion of sputum samples for 10, 15 and 20 minutes yielded mycobacterial growth in 72 (48%), 81 (54%) and 68 (45%) of the digested samples respectively after incubation in the MGIT system.

**Conclusions**: In routine laboratory conditions of a resource-limited setting, our study has demonstrated the superiority of fluorescence microscopy over the conventional ZN technique. Digestion of sputum samples for 15minutes yielded more positive cultures



**Keywords**: Tuberculosis, Microbiology, Respiratory infections

**Word count**: 3,399

**Article focus**:

1. What is the optimal performance of the new light-emitting diode (LED) florescence microscopy in routine conditions of a resource limited setting and how does it compare to the traditional light microscopy using Ziehl Neelsen (ZN) technique for AFB smear microscopy?
2. What is the optimal digestion time for sputum samples for maximum yield of positive cultures in routine conditions of a resource limited setting?
3. Despite the operational challenges of resource limited settings flourescence microscopy has better optimal performance compared to the traditional light microscopy using Ziehl Neelsen (ZN) technique for AFB smear microscopy.

**Significance**:

1. Most of the settings with highest burden of Tuberculosis in the world are limited in resource needed to effectively detect and manage cases of the disease.
2. The WHO has recommended the phasing out of the simple, inexpensive but less sensitive ZN technique with the more sensitive florescence technique for better TB detection in all settings.
3. Evaluating the performance of the more sensitive florescence microscopy against the less sensitive ZN microscopy in unaltered working conditions of resource limited settings is relevant for effective TB control.

**Strength and Limitations**:

1. The design of this study allows test performance assessment under routine (real) resource constrain settings.
2. Blinding the microscopist on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of direct smear results
3. However, our inability to obtain participant HIV status deprived us the ability to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures.

## Introduction

Globally, an estimated 1.4 million death occurred in 2011 as a result of infection with tuberculosis, one-fourth of the death was associated with HIV infection and most of it in resource limited settings (1) where the burden of HIV infection is high. In Africa, TB is the leading cause of death among HIV infected persons as the continent harbors eighty percent of the world HIV-TB cases (2). Nigeria and the Republic of South Africa are among the top five TB burden nations of the world. Sensitive, specific, and inexpensive point-of-care diagnostic tools for rapid TB detection are lacking posing challenges to the optimal diagnosis of TB in resource limited settings (RLS), particularly among persons with HIV infections whose sputum specimen often lack sufficient quantity of acid-fast bacilli (AFB) detectable on routine smear examination. (3, 4)

The simplicity, inexpensiveness and predictive power of the Ziehl Neelsen sputum smear microscopy make it the applicable laboratory diagnostic tool of choice for tuberculosis in resource limited settings. (5) A properly done positive sputum-smear is highly predictive of active TB (6, 7). Unfortunately, the sensitivity of sputum-smear microscopy is low and its performance is often affected by lack of proper maintenance of equipment; trained manpower; and quality assurance system in settings where resources are scarce. (8) Even in centers with good quality assurance measures, smear microscopy detects only 40-50% of TB cases compared to about 80% yield by culture (9). However, it remains the only available tool for bacteriologic diagnosis of TB in most RLS. Culture techniques are highly sensitive and specific, but the cost, technical complexity and time delay before results are available make culture not scalable for rapid detection and treatment of tuberculosis. Recent advances have made it possible to use molecular technology to detect Mycobacterial deoxyribonucleic acid (DNA) with great accuracy. However these technologies still require a fairly developed laboratory with a reliable source of electricity.

Detection of smear positive cases is the priority in TB control programs. These cases are infectious and contribute substantially to the transmission of the disease. (10, 11) In recent years, several new laboratory techniques have been developed to significantly improve the ability to diagnose active TB in populations. Fluorescence microscopy improves the sensitivity of sputum-smear detection of TB and has good specificity relative to the conventional ZN method. Inexpensive light-emitting diode-based (LED) fluorescence

microscopes have been developed to make microscopy of smears stained by Auramine fluorescence dye easier, cheaper and faster with potentials for scalability to remote clinics in RLS. (5, 12-14).

This study compared the performance of the standard but more expensive mercury vapor lamp (FM) fluorescence microscope; the newer, less expensive LED based CysCope® (CY) (Partec, Görlitz, Germany); and the Primo Star iLED (PiLED) (Carl Zeiss Inc. Germany) fluorescence microscopes in comparison to the conventional light ( ZN) microscope against the gold (reference) standard of liquid culture in automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems) for the detection of AFB from sputum samples of patients with clinical pulmonary tuberculosis (TB). In addition, we evaluated the optimal digestion time with *Mycoprep (4%NaOH-1%NLAC and 2.9% sodium citrate)* for liquid culture in this population. Prolonged decontamination time is reported to reduce the number of viable bacilli and different digestion methods yield variable number of positive cultures (15-17).

## Methods

*Study population*

Three sputum samples (spot-morning-spot) were collected from 150 TB treatment naïve, consecutive patients with clinical diagnosis of pulmonary tuberculosis at the National TB and Leprosy Training Center (NTBLTC), Zaria, Nigeria. Routine and referred cases of TB from northern Nigerian region receive TB treatment and care at this facility. The spot-morning-spot sample collection was the standard routine for the diagnosis of TB in Nigeria at the time of the study. The flow chart in **Figure 1** provides an overview of the study design. Ethical review was waived because in the opinion of the study center review committee there was no potential risk to participants' safety, privacy or confidentiality since there was no formal contact between investigators and participants either directly (interview, questionnaires, etc.) or indirectly (medical records, personal identifiers etc.). The sputum specimens provided for routine clinical care services were completely anonymized before they were analyzed for the study and there was no risk that the pooled samples can be de-anonymized through data linkages. This study was conducted from July to September of 2009.

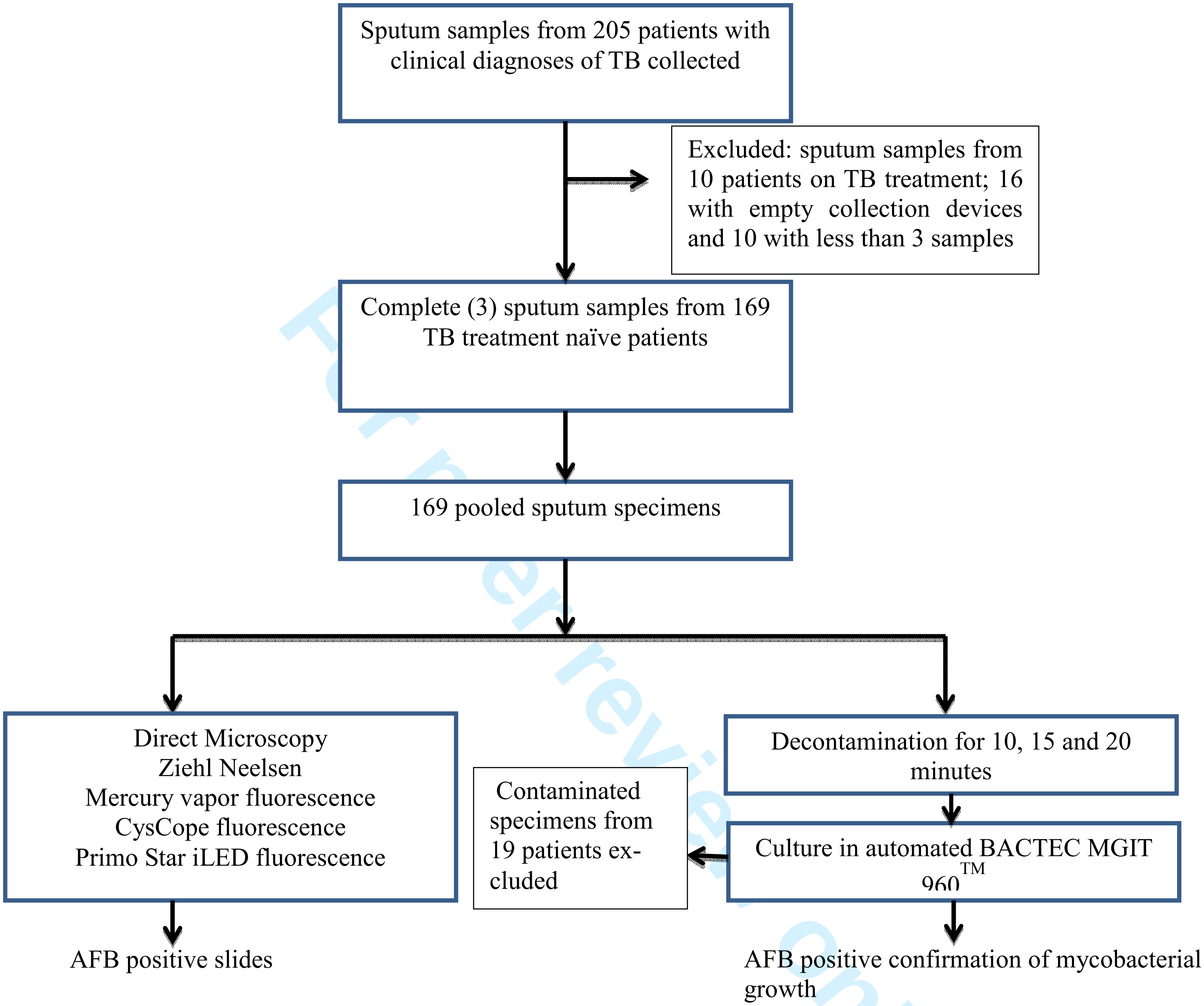


**Figure 1**. Flow chart for the study design involving 450 sputum samples from 150 patients with clinical TB

*Smear Microscopy*

The three sputum samples from each patient were pooled. Four direct smears were made from each of the pooled sputum samples with applicator sticks on clean grease-free slides; measuring 2 cm x 3 cm, and not too thick. The first slide was stained with Ziehl Neelsen (ZN) stain according to the World Health Organization (WHO) recommended protocol described elsewhere. (18) Briefly, the smear was air dried and fixed by gently passing it over a flame 2-3 times. One percent strong carbol-fuchsin was applied to the slide and heated with a Bunsen flame intermittently 3 times and allowed to stain for 15 minutes. The stain was rinsed off with tap water and 3% acid alcohol was applied on the smear for 3 minutes. Methylene blue (0.3%) was then added for up to 1 minute and rinsed off with tap water. Positive and negative controls were included in the process. Slide examination was done with light microscope at 1,000x magnification and the AFBs identified were graded according to the International Union against Tuberculosis and Lung Disease (IUATLD) and the WHO smear grading scale. (19)

The second, third and fourth slides were air-dried, heat-fixed and stained with Auramine O-phenol for 15 minutes rinsed with tap water, decolorized with 1 percent acid alcohol for 2 minutes then rinsed with water and counterstained with 0.1 percent potassium permanganate for 2 minutes. The slides were rinsed with water then allowed to dry in air before examination under FM, CY and PiLED microscopes respectively. The AFBs were visualized and graded at magnifications of 200× and 400× according to a protocol describe by Lehman (20). A total of 100 microscopic fields were examined before a slide is recorded as positive (if at least an AFB was seen) or negative (if no AFB was seen). Positive smears were graded as scanty (actual) if there were: 1-9 AFBs in at least 100 fields; +1: 10-99 in 100 fields; +2: 1-10 per field in at least 50 fields; and +3: if there were more than 10 per field in at least 20 fields. Absence of any AFB was graded as negative smear. Each slide was examined by three independent readers to ascertain the presence of AFB and grade positive smears. The slide readers were blinded on the clinical and laboratory diagnoses of the subjects whose samples were studied.

*Specimen culture*

Each of the 150 pooled sputum specimens were decontaminated with BD Mycoprep$^{TM}$ (Beckton Dickinson Diagnostic Systems, Sparks, Maryland, USA) which consists of 1% N-acetyl-L-cysteine (NALC), 4% sodium hydroxide and 2.9% sodium citrate. An equal amount of Mycoprep was added to the sputum, homogenized and

allowed to act for 10, 15 and 20 minutes respectively. After the respective digestion period, phosphate buffered saline was added to stop the digestion and decontamination reaction. This was then centrifuged under refrigerated conditions with uniform centrifugation time of 15 minutes and a Relative Centrifugal force (RCF) of 3000 x g. The supernatants were discarded and the sediments re-suspended with 1 ml of phosphate buffered saline to make a final volume of 2 ml. Then 0.5 ml of each was inoculated into Mycobacterium Growth Indicator Tubes (MGIT) and incubated in the automated BACTEC MGIT 960™ machine (Becton Dickinson Diagnostic Instrument Systems), which monitors growth. Positive and negative control culture tubes were also set up along each test using *Mycobacterium tuberculosis* H37Ra (ATCC 25177 – attenuated strain) and sterile distilled water respectively.

The cultures that indicated positive growth were removed from the machine and the presence of AFB confirmed by ZN smear microscopy. Those that do not indicate growth after 42 days were classified as negatives as recommended by the manufacturer and as was the standard practice. All cultures were done at Zankli Research laboratory while all microscopy were performed at the NTBLTC, Zaria to minimize interference with the outcome of culture or direct microscopy results.

*Statistical analysis*

The raw data generated from the smear microscopic examinations of the 150 matched samples by the four different microscopes as well as the data generated after same samples were cultured following decontaminations (digestions) at 10, 15 and 20 minutes were organized into cell counts in which descriptive values were provided along with frequencies and proportions. Measures of accuracy for the different smear examinations: sensitivity, specificity, positive and negative predictive values were evaluated through decision matrix tables while concordance and disagreements between the microscopic examination by each of the three fluorescence microscopes and the conventional light (ZN) microscope based on the final classification of samples by the gold standards (outcomes MGIT cultures) were examined in matched sample tables for positive and negative final outcomes as previously recommended for comparative analysis of diagnostic procedures performed on same samples (patients) (21). Comparison between the fluorescence microscopies to the conventional ZN microscopy for differences in performance together with their 95% confidence intervals was done using the McNemar's test. Significance of association was set at 0.05 alpha and all probabilities were two tailed.

**Results**

Of the 150 matched (paired) samples, AFBs were detected in: 44 (29%) of the ZN smears examined under light microscope; 60 (40%) of the AR smears examined with FM; 49 (33%) of the AR smears examined with CY; and 64 (44%) AR smears examined with PiLED fluorescence microscopes. MGIT cultures of the 150 specimen yielded 72 (48%), 81 (54%) and 68 (45%) AFB confirmed growths after decontamination for 10 (A), 15 (B) and 20 (C) minutes respectively prior to incubation.).

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Among the positive AFB tests graded, ZN light microscopy produced the highest proportion of samples with scanty AFBs on examination 21/44 (48%), followed by PiLED fluorescence microscopy 12/64 (19%), then CY 8/49 (16%) and FM had the least scanty AFB positive counts 3/60 (5%). However, after the samples with scanty positive AFBs where incubated in A, B and C MGIT culture groups, the proportions of confirmed mycobacterial growths in the sequence above were as follows: (i) ZN: 14/21 (67%), 9/21 (42%) and 11/21 (52%); (ii) PiLED: 5/12 (42%), 7/12 (58%) and 5/12 (42%); (iii) CY: 6/8 (75%), 4/8 (50%) and 3/8 (38%); lastly (iv) FM: 0/3 (0%), 1/3 (33%) and 1/3 (33%). A summary of the positive AFB outcomes from the direct smear examinations by microcopy types, grades and the proportions of graded smears confirmed by the MGIT cultures were provided in **Table 1**.

**Table 1**. Proportions of positive graded direct smears confirmed by cultured specimen decontaminated at 10 (A), 15 (B) and 20(C) minutes obtained from 150 matched samples

| Direct smear (DS) | Grade | Frequency | MGIT cultured specimen (Gold standards) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A | | B | | C | |
| | | | n | % | n | % | n | % |
| ZN | scanty | 21 | 14 | 66.7 | 9 | 42.1 | 11 | 52.4 |
| | +1 | 13 | 12 | 92.3 | 11 | 84.6 | 10 | 76.9 |
| | +2 | 5 | 5 | 100.0 | 4 | 80.0 | 5 | 100.0 |
| | +3 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| FM | Scanty | 3 | 0 | 0.0 | 1 | 33.3 | 1 | 33.3 |
| | +1 | 34 | 24 | 70.6 | 19 | 55.9 | 17 | 50.0 |
| | +2 | 18 | 16 | 88.9 | 12 | 66.7 | 13 | 72.2 |
| | +3 | 5 | 4 | 80.0 | 4 | 80.0 | 4 | 80.0 |
| CY | Scanty | 8 | 6 | 75.0 | 4 | 50.0 | 3 | 37.5 |
| | +1 | 30 | 22 | 73.3 | 18 | 60.0 | 19 | 63.3 |
| | +2 | 5 | 5 | 100.0 | 5 | 100.0 | 5 | 100.0 |
| | +3 | 6 | 6 | 100.0 | 6 | 100.0 | 6 | 100.0 |
| PiLED | Scanty | 12 | 5 | 41.7 | 7 | 58.3 | 5 | 41.7 |
| | +1 | 36 | 22 | 61.1 | 20 | 55.6 | 18 | 50.0 |
| | +2 | 13 | 13 | 100.0 | 10 | 76.9 | 13 | 100.0 |
| | +3 | 3 | 3 | 100.0 | 2 | 66.7 | 3 | 100.0 |

ZN: Ziehl Neelsen microscopy using the conventional light microscope
FM: Fluorescence microscopy with mercury vapor lamp microscope
CY: Fluorescence microscopy with CysCope light emitting diode microscope
PiLED: Fluorescence microscopy with Primo Star iLED light emitting diode microscope



A comparison of the direct smear tests results with the final culture outcomes to measure the performances of the direct smear techniques against the gold standards showed that of the 44 AFB positive samples identified by the ZN microscopy; 36 (81.8%), 29 (65.9%) and 31 (70.5%) were culture confirmed after 10, 15 and 20 minutes decontamination respectively. Similarly, of the 60, 49 and 64 AFB positive samples identified by the FM, CY and PiLED microscopy techniques the frequencies and proportions of the culture confirmed samples were: 44 (73.3%) 36 (60.0%) and 35 (58.3%) for FM; 39 (79.6%), 33 (67.3%) and 33 (67.3%) for CY;

and 43 (67.2%) 39 (56.3%) and 39 (56.3%) for PiLED at decontamination times of 10, 15 and 20 minutes respectively. There were near identical frequency distributions for the true positive (TP), false positive (FP), false negative (FN) and true negative (TN) for the ZN and CY microscopy techniques while the FM microscopy yielded a pattern of distribution similar to that of PiLED technique as shown in the decision matrix of **Table 2**.

**Table 2**. Decision matrix for the evaluation of performance of the direct smear methods against the different gold standards for the 150 matched samples

| Direct Smear (DS) | DS Outcome | Gold standards: MGIT cultures | | | | | |
|---|---|---|---|---|---|---|---|
| | | A : culture positive =72 | | B: culture positive =81 | | C: culture positive = 68 | |
| | | P | N | P | N | P | N |
| ZN | P | 36 | 08 | 29 | 15 | 31 | 13 |
| DS+ = 44 | N | 36 | 70 | 52 | 54 | 37 | 69 |
| FM | P | 44 | 16 | 36 | 24 | 35 | 25 |
| DS+ =60 | N | 28 | 62 | 45 | 45 | 33 | 57 |
| CY | P | 39 | 10 | 33 | 16 | 33 | 16 |
| DS+ =49 | N | 33 | 68 | 48 | 53 | 35 | 66 |
| PiLED | P | 43 | 21 | 39 | 25 | 39 | 25 |
| DS+ =64 | N | 29 | 57 | 42 | 44 | 29 | 57 |

Culture specimen decontaminated by NaOH-NALC : A for 10 minutes; B for 15minutes; C for 20 minutes
DS+ = number of samples (out of 150 samples) that were AFB positive by direct smear
P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor fluorescence microscopy; CY=CysCope fluorescence microscopy and PiLED= Primo Star iLED fluorescence microscopy

The levels of agreement and differences between the convention ZN microscopy and each of the fluorescence microscopy techniques with respect to the final culture results were demonstrated by the concordance and discordance cells displayed in **Table 3**. Cells were discordant if the outcomes of the two techniques disagreed otherwise they were concordant. The statistical significance of the difference in the performance of the techniques compared was derived from the discordant cell. The sum of the frequencies of the discordant cells for example that explained the differences between ZN and CY were 7, 4 and 2 for the positive culture results which showed less consistent divergence compared to 6, 6, and 7 for the negative culture results of the same

techniques. The sum of the discordant frequencies between ZN and FM, and between ZN and PiLED were higher relative to those between ZN and CY.

**Table 3**: Matched sample frequency cells for the agreement and discordance between the standard of care (ZN smear) and FM, CY and PiLED smears on the culture positive and negative specimens

| | | | FM | | CY | | PiLED | |
|---|---|---|---|---|---|---|---|---|
| Culture Positive | | | P | N | P | N | P | N |
| A: n= 72 | ZN | P | 35 | 1 | 34 | 2 | 35 | 1 |
| | | N | 9 | 27 | 05 | 31 | 08 | 28 |
| B: n= 81 | ZN | P | 29 | 0 | 29 | 0 | 29 | 0 |
| | | N | 7 | 45 | 4 | 48 | 10 | 42 |
| C: n= 68 | ZN | P | 29 | 2 | 31 | 0 | 29 | 2 |
| | | N | 6 | 31 | 2 | 35 | 10 | 27 |
| Culture Negative | | | | | | | | |
| A: n= 78 | ZN | P | 6 | 2 | 7 | 2 | 9 | 0 |
| | | N | 7 | 63 | 4 | 65 | 12 | 57 |
| B: n= 69 | ZN | P | 11 | 1 | 6 | 2 | 14 | 0 |
| | | N | 10 | 47 | 4 | 57 | 10 | 45 |
| C: n= 82 | ZN | P | 11 | 1 | 10 | 1 | 13 | 0 |
| | | N | 12 | 58 | 6 | 65 | 10 | 59 |

P= positive; N = Negative; ZN: Ziehl Neelsen microscopy; FM= mercury vapor fluorescence microscopy; CY=CysCope fluorescence microscopy and PiLED= Primo Star iLED fluorescence microscopy
A: cultured samples decontaminated for 10 minutes; B: cultured samples decontaminated for 15 minutes; C: cultured samples decontaminated for 20 minutes

The ZN technique was consistently less sensitive compared to any of the fluorescence techniques against any of the gold standards. The FM fluorescence technique was: 11.1%, 8.6% and 5.9%; CY method was: 4.2%, 4.9% and 3.1% while PiLED was 9.7%, 12.3%, and 11.8% more sensitive than ZN microscopy for the different digestion times of 10, 15 and 20 minutes respectively ( $P_{fm}$= 0.027, 0.023, 0.289; $P_{cy}$ = 0.450, 0.134, 0.479 and $P_{iled}$ =0.046, 0.004, 0.043). Conversely, the FM was: 10.2%, 13.1% and 14.6%; CY was: 2.5% 2.5% and 3.6%; and PiLED was: 16.6%, 1.4% and 14.6% less specific compared to the ZN microscopy for the same digestion times above ($Pfm$= 0.182, 0.016, 0.006; $P_{cy}$ = 0.684, 0.684, 0.131 and $P_{iled}$ =0.006, 0.131, and 0.004). They were also less predictive than the ZN technique (**Table 4**).

**Table 4**. Summary measures of performance of the direct smear diagnostic tests for 150 matched samples and the statistical differences in sensitivity and specificity of FM, CY and PiLED in comparison to ZN

| Gold Stand | Direct Smear | Sn | Sp | PPV | NPV | SnD [95% CI] | p-value* | SpD [95% CI] | p-value* |
|---|---|---|---|---|---|---|---|---|---|
| A | ZN | 50.0 | 89.7 | 81.8 | 66.0 | | | | |
| | FM | 61.1 | 79.5 | 73.3 | 68.9 | 11.1 [3.0, 19.0] | 0.027 | 10.2 [2.5, 17.4] | 0.182 |
| | CY | 54.2 | 87.2 | 79.6 | 67.3 | 4.2 [-2.9, 11.3] | 0.450 | 2.5 [-3.6, 8.6] | 0.684 |
| | PiLED | 59.7 | 73.1 | 67.2 | 66.3 | 9.7 [1.9, 17.5] | 0.046 | 16.6 [8.6, 24.6] | 0.001 |
| B | ZN | 35.8 | 78.3 | 65.9 | 50.9 | | | | |
| | FM | 44.4 | 65.2 | 60.0 | 50.0 | 8.6 [2.4, 14.8] | 0.023 | 13.1 [3.9, 22.1] | 0.016 |
| | CY | 40.7 | 76.8 | 67.3 | 52.5 | 4.9 [0.2, 9.6] | 0.134 | 2.5 [-4.4, 9.4] | 0.684 |
| | PiLED | 48.1 | 63.8 | 60.9 | 51.2 | 12.3 [4.9, 19.6] | 0.004 | 1.4 [-5.5, 7.9] | 0.683 |
| C | ZN | 45.6 | 84.1 | 70.5 | 65.1 | | | | |
| | FM | 51.5 | 69.5 | 58.3 | 63.3 | 5.9 [-2.1, 13.9] | 0.289 | 14.6 [6.6, 22.6] | 0.006 |
| | CY | 48.5 | 80.5 | 67.3 | 65.3 | 3.1 [-0.9, 7.1] | 0.479 | 3.6 [-2.6, 9.8] | 0.131 |
| | PiLED | 57.4 | 69.5 | 60.9 | 66.3 | 11.8 [2.2, 21.4] | 0.043 | 14.6 [7.5, 21.7] | 0.004 |

Sn: Sensitivity; Sp: Specificity; PPV: positive predictive value; NPV; Negative predictive value
SnD: Sensitivity difference; percentage points above ZN performance
SpD: Specificity difference; percentage points below ZN performance
CI: Confidence Interval; *: Chi-square p-value generated from McNemar's test

**Discussion**

The findings of this study further confirm the previously reported superior performance of fluorescence microscopy over the conventional ZN technique for AFB detection (22-24). The direct smear staining sensitivities of FM and PiLED fluorescence techniques are on the average 10% more sensitive than the standard of care (ZN method). The reverse is however true for specificity, the fluorescence methods are less specific than ZN by similar percentage points and these differences are statistically significant. Similar trends of high sensitivity and low specificity were observed in the performance of fluorescence microscopy in comparison to the conventional Ziehl Neelsen technique (24-26) Within the fluorescence techniques FM and PiLED have similar performance measures with little or no differences which is in agreement with the reported findings by Marais et al.(27) The CY technique appears to be less sensitive compared to FM and PiLED but on the average have 4% staining sensitivity advantage over the conventional ZN. Similar findings were reported from a resource constrained setting with comparable optimal technical conditions in which such differences were found to be marginally significant statistically. (20). However, both CY and ZN staining techniques have higher specificities compared to FM and PiLED across all the three reference (gold) standards used.

Although less sensitive, the ZN staining seems to be the most predictive: a positive test has the highest chance of being a true case of TB while a negative test is less likely to harbor the disease. The CY has similar predictive probabilities; however, these measures are affected by disease burden. In high prevalence settings, the positive predictive value of a test increases because it is more likely that those who test positive truly have disease than if the test was performed in settings with low prevalence. Likewise, in settings with low prevalence it is more likely that those who test negative truly do not have the disease. In contrast, sensitivity and specificity measures are less susceptible to influence by disease prevalence. The search for an optimal tuberculosis diagnostic test is motivated by the unacceptably low sensitivity of the conventional ZN smear microscopy over which fluorescence microscopy has shown consistent superiority. In this setting, the TB prevalence is high and may be driven by HIV. Priority should therefore be given to sensitivity over predictive value since failure to detect TB in patients co-infected with HIV is more likely to increase morbidity and mortality among the HIV infected persons than false detection of TB.

In addition to improved TB case detection in samples with low-density bacilli, which is aided by its low-power objective, previous studies reported that fluorescence microscopy requires less than 25% of the time taken to read slides using the conventional ZN technique-meaning a microscopist can examine 4 times the number of slides per day with fluorescence technique.(20, 28) However, the use of mercury vapor or halogen lamp as the source of light in the standard fluorescence microscopy technique (FM) is expensive and requires frequent replacement because of their shorter life span making it economically inefficient for use in resource constrained settings. The LED based microscopes (CY and PiLED) on the other hand are structurally built to overcome the hard operational environment of RLS where continuous supply of electricity, sufficient equipment and trained personnel are a frequent challenge. The LED microscopes use lamps that are inexpensive with much longer life span. They are also simple to operate; and no dark room required.

The scanty-positive findings are more commonly seen with ZN technique and least with the FM. This could be attributed to the low-power objective of the fluorescence techniques that allow the field to be seen larger than in the conventional ZN light microscopy. Some of the samples studied may have come from HIV co-infected patients with low-density bacilli yielding scanty AFBs on the conventional ZN microscopy earning a grade of 1+ or more when viewed under the fluorescence techniques. However, the fluorescence techniques recorded the highest numbers of false positives compared to the ZN judging from the reported frequencies in the decision matrix table. This is expected because the low-threshold of the fluorescence techniques derived from their low-power objective could allow naturally fluorescence particles present in the sputum, certain spores, fungi to appear AFB positive which may otherwise be negative on ZN microscopy. These and the fact that significant proportion of the samples studied may have come from HIV co-infected patients could also explain the particularly low specificity of the PiLED technique. Associated HIV infection was believed to account for low specificity of LED-FM microscopy compared to the conventional ZN microscopy in two previous studies in settings with high burden of the disease (29, 30)

As expected, the digestion and decontamination with n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) produces the highest yield of AFB in the sputum exposed to the reagent for 15 minutes. This shows that the manufacturer's recommended exposure time of 15 minutes works well in the hard operational environment of a resource limited setting. The 10 minutes alternative yielded a little less AFB than the 15 minutes duration probably because the time is not enough to allow complete digestion of the thick sputum which allows the release of mycobacteria and their subsequent concentration by centrifugation. The 20 minutes exposure on the other hand might have been prolonged beyond the optimal duration necessary and the toxic effect of the reagent kills not only the contaminating normal flora that may overgrow in cultures making it hard to detect the presence of the mycobacteria but also the mycobacteria itself. The success of this process is dependent on the concentration of the reagent, the exposure time, centrifugation and the temperature selected. In this case all other factors were kept constant and only the exposure time was altered.

*Some of the reasons for the unusually high proportion of AFB positive, culture negative specimen could possibly be that some of the patients contrary to their claim have actually been on TB treatment at presentation. This may have adversely hindered the bacilli ability to grow, or more importantly in this case, the bacilli may have been killed by the excessive decontamination in the samples decontaminated for up to 20 minutes especially among cases with paucibacillary disease due to HIV co-infection. These patients may likely test positive to both smear microscopy and culture in repeat examinations with optimal decontamination time. Fungal infections are also not uncommon in the study area and together with some artefacts may have added to the high frequency of the smear positive, culture negative findings.*

The design of this study allows test performance assessment under routine (real) resource constrain settings. The point estimates obtained are more likely to represent the expected outcomes in similar settings with high prevalence of TB. Blinding the readers on the clinical or laboratory diagnosis associated with the samples examined minimized bias in the interpretation of the direct smear results. However, our study had a few limitations: We did not obtain participants' HIV status and therefore unable to measure its impact on the performance of the techniques and the culture outcomes at different decontamination exposures. The time taken to read slides was also not reflected in the analysis because only estimates of the time taken to read the slides were recorded since the different technicians that read the slides had different proficiency levels and some accurately read the slides much faster than others.

In summary, the high sensitivity for AFB detection shown by the inexpensive light-emitting diode based Primo Star iLED fluorescence microscope in the hard operational conditions of a resource limited setting underscores the potentials for its scalability to remote clinics in similar settings.. This will increase TB detection and improve TB treatment uptakes in high burden settings with scarce resources. The optimal exposure duration for decontamination of sputum samples prior to examination and culture using the n-acetyl-L-cysteine (NALC)- sodium hydroxide (NaOH) reagent in this setting is 15 minutes.

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## References

1. De Cock KM, Simone PM, Davison V, Slutsker L. The new global health. Emerg Infect Dis. 2013 Aug;19(8):1192-7.
2. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. [Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.
Review]. 2003 May 12;163(9):1009-21.
3. Young DB, Perkins MD, Duncan K, Barry CE, 3rd. Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest. [Research Support, Non-U.S. Gov't
Review]. 2008 Apr;118(4):1255-65.
4. Getahun H, Harrington M, O'Brien R, Nunn P. Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. Lancet. [Review]. 2007 Jun 16;369(9578):2042-9.
5. Somoskovi A, Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M. Lessons from a proficiency testing event for acid-fast microscopy. Chest. [Research Support, U.S. Gov't, P.H.S.]. 2001 Jul;120(1):250-7.
6. Van Deun A, Salim AH, Cooreman E, Hossain MA, Rema A, Chambugonj N, et al. Optimal tuberculosis case detection by direct sputum smear microscopy: how much better is more? Int J Tuberc Lung Dis. [Evaluation Studies]. 2002 Mar;6(3):222-30.
7. Cole EC, Cook CE. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. Am J Infect Control. [Review]. 1998 Aug;26(4):453-64.
8. Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin Microbiol Rev. [Review]. 2011 Apr;24(2):314-50.
9. Monkongdee P, McCarthy KD, Cain KP, Tasaneeyapan T, Nguyen HD, Nguyen TN, et al. Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. Am J Respir Crit Care Med. [Evaluation Studies
Research Support, U.S. Gov't, Non-P.H.S.]. 2009 Nov 1;180(9):903-8.

10. Cheng S, Chen W, Yang Y, Chu P, Liu X, Zhao M, et al. Effect of Diagnostic and Treatment Delay on the Risk of Tuberculosis Transmission in Shenzhen, China: An Observational Cohort Study, 1993-2010. PLoS One. 2013;8(6):e67516.
11. Tadesse T, Demissie M, Berhane Y, Kebede Y, Abebe M. Incidence of smear-positive tuberculosis in Dabat, northern Ethiopia. Int J Tuberc Lung Dis. [Research Support, U.S. Gov't, Non-P.H.S.]. 2013 May;17(5):630-5.
12. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. [Comparative Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.
Review]. 2006 Sep;6(9):570-81.
13. Hooja S, Pal N, Malhotra B, Goyal S, Kumar V, Vyas L. Comparison of Ziehl Neelsen & Auramine O staining methods on direct and concentrated smears in clinical specimens. Indian J Tuberc. [Comparative Study]. 2011 Apr;58(2):72-6.
14. Prasanthi K, Kumari AR. Efficacy of fluorochrome stain in the diagnosis of pulmonary tuberculosis co-infected with HIV. Indian J Med Microbiol. [Comparative Study]. 2005 Jul;23(3):179-81.
15. Abe C. [Standardization of laboratory tests for tuberculosis and their proficiency testing]. Kekkaku. [Review]. 2003 Aug;78(8):541-51.
16. Allen BW. Comparison of three methods for decontamination of faeces for isolation of Mycobacterium tuberculosis. Tubercle. [Comparative Study]. 1991 Sep;72(3):214-7.
17. Krasnow I, Wayne LG. Comparison of methods for tuberculosis bacteriology. Appl Microbiol. [Comparative Study]. 1969 Nov;18(5):915-7.
18. Selvakumar N, Rahman F, Rajasekaran S, Narayanan PR, Frieden TR. Inefficiency of 0.3% carbol fuchsin in ziehl-neelsen staining for detecting acid-fast bacilli. J Clin Microbiol. [Comparative Study
Evaluation Studies
Research Support, Non-U.S. Gov't]. 2002 Aug;40(8):3041-3.
19. http://www.uphs.upenn.edu/bugdrug/antibiotic_manual/IUATLD_afb%20microscopy_guide.pdf. Accessed last on December 25.
20. Lehman LG, Ngapmen Yamadji AL, Ngo Sack F, Bilong Bilong CF. The CyScope(R) fluorescence microscope, a reliable tool for tuberculosis diagnosis in resource-limited settings. Am J Trop Med Hyg. [Research Support, Non-U.S. Gov't]. 2010 Oct;83(4):906-8.
21. Hawass NE. Comparing the sensitivities and specificities of two diagnostic procedures performed on the same group of patients. Br J Radiol. [Comparative Study]. 1997 Apr;70(832):360-6.
22. Hung NV, Sy DN, Anthony RM, Cobelens FG, van Soolingen D. Fluorescence microscopy for tuberculosis diagnosis. Lancet Infect Dis. [Comment
Letter]. 2007 Apr;7(4):238-9; author reply 9-40.
23. Van Deun A, Chonde TM, Gumusboga M, Rienthong S. Performance and acceptability of the FluoLED Easy module for tuberculosis fluorescence microscopy. Int J Tuberc Lung Dis. [Comparative Study
Evaluation Studies
Research Support, U.S. Gov't, Non-P.H.S.]. 2008 Sep;12(9):1009-14.
24. Ulukanligil M, Aslan G, Tasci S. A comparative study on the different staining methods and number of specimens for the detection of acid fast bacilli. Mem Inst Oswaldo Cruz. [Comparative Study]. 2000 Nov-Dec;95(6):855-8.

25. Cuevas LE, Al-Sonboli N, Lawson L, Yassin MA, Arbide I, Al-Aghbari N, et al. LED fluorescence microscopy for the diagnosis of pulmonary tuberculosis: a multi-country cross-sectional evaluation. PLoS Med. [Controlled Clinical Trial
Multicenter Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.]. 2011 Jul;8(7):e1001057.
26. Habtamu M, van den Boogaard J, Ndaro A, Buretta R, Irongo CF, Lega DA, et al. Light-emitting diode with various sputum smear preparation techniques to diagnose tuberculosis. Int J Tuberc Lung Dis. [Comparative Study
Research Support, Non-U.S. Gov't]. 2012;16(3):402-7.
27. Marais BJ, Brittle W, Painczyk K, Hesseling AC, Beyers N, Wasserman E, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clin Infect Dis. [Comparative Study
Evaluation Studies]. 2008 Jul 15;47(2):203-7.
28. Ba F, Rieder HL. A comparison of fluorescence microscopy with the Ziehl-Neelsen technique in the examination of sputum for acid-fast bacilli. Int J Tuberc Lung Dis. [Comparative Study]. 1999 Dec;3(12):1101-5.
29. Cattamanchi A, Davis JL, Worodria W, den Boon S, Yoo S, Matovu J, et al. Sensitivity and specificity of fluorescence microscopy for diagnosing pulmonary tuberculosis in a high HIV prevalence setting. Int J Tuberc Lung Dis. [Comparative Study
Evaluation Studies
Research Support, N.I.H., Extramural]. 2009 Sep;13(9):1130-6.
30. Chaidir L, Parwati I, Annisa J, Muhsinin S, Meilana I, Alisjahbana B, et al. Implementation of LED fluorescence microscopy for diagnosis of pulmonary and HIV-associated tuberculosis in a hospital setting in Indonesia. PLoS One. [Research Support, Non-U.S. Gov't]. 2013;8(4):e61727.

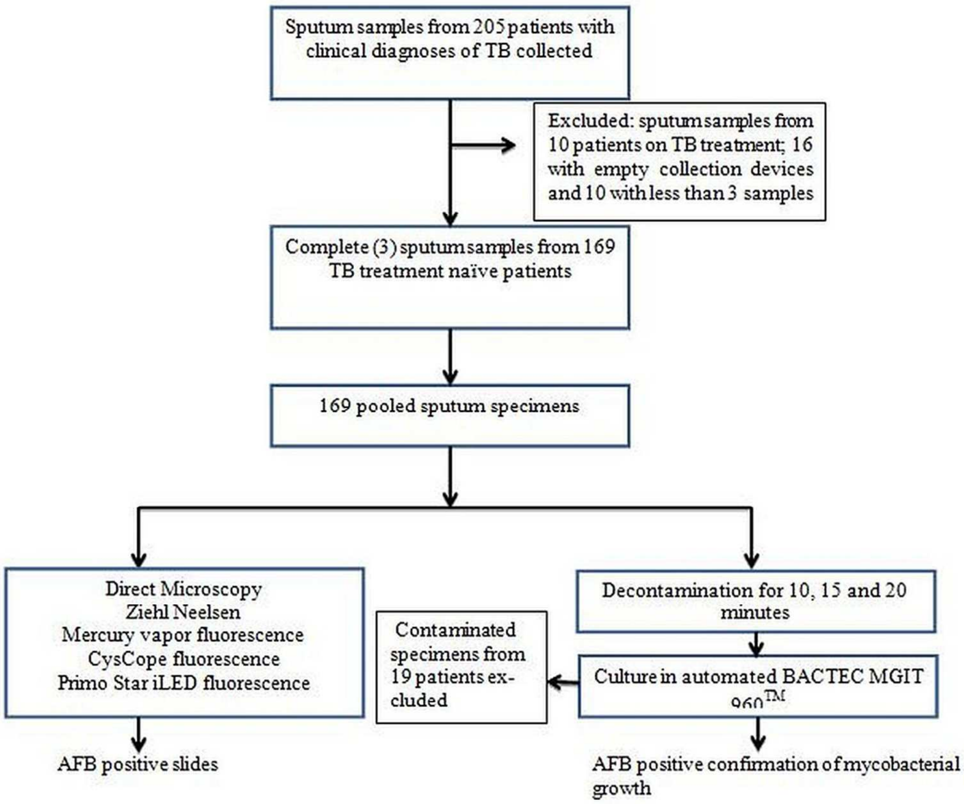


**Figure 1**. Flow chart for the study design involving 450 sputum samples from 150 patients with clinical TB

Figure 1. Flow chart for the study design involving 450 sputum samples from 150 patients with clinical TB

92x90mm (300 x 300 DPI)

**Checklist for optimizing tuberculosis detection manuscript submitted to BMJ open**

| Section and Topic | Item # | | On page # |
|---|---|---|---|
| TITLE/ABSTRACT/ KEYWORDS | 1 | *Optimizing Mycobacterium tuberculosis detection in resource limited settings*: a comparison between different types of florescence smear microscopy techniques with the traditional Zeal Nielsen's (ZN) smear microscopy under routine working conditions of a resource limited setting. | 1 |
| INTRODUCTION | 2 | The diagnostic accuracies of the standard but more expensive mercury vapor lamp (FM) florescence microscope; the newer, less expensive LED based CyScope® (CY); and the Primo Star iLED (PiLED) florescence microscopes were compared to that of the conventional light ( ZN) microscope against the gold (reference) standard of liquid culture in automated BACTEC MGIT 960™ machine | 2 |
| METHODS | | | |
| *Participants* | 3 | 450 sputum samples from 150 new patients with clinical diagnosis of pulmonary tuberculosis were studied from the main tuberculosis case referral center in northern Nigeria. | 3 |
| | 4 | Participant recruitment: sputum samples from new patients with clinical diagnosis of pulmonary tuberculosis based on presenting symptoms independent of the outcome of the routine (standard of care TB test) were selected for this study. | 3 |
| | 5 | Participant sampling: Sputum samples of consecutive new but de-identified patients with clinical diagnosis of pulmonary tuberculosis were sampled. | 3 |
| | 6 | Data collection: This was a cross-sectional study where three sputum samples collected from patients over 24 hour period (spot-morning-spot) for the purpose of routine clinical care were used. | 3 |
| *Test methods* | 7 | The reference standard was sputum culture in Mycobacterium Growth Indicator Tubes (MGIT) and incubated in the automated BACTEC MGIT 960™ machine. Liquid cultures are more sensitive, fast and reliable techniques for the diagnosis of pulmonary tuberculosis | 4 |
| | 8 | The three sputum samples collected from each patient were pooled into a single specimen. Four direct smears were made from each of the pooled specimen. The first slide was stained with Ziehl Neelsen (ZN) while the second, third and fourth slides were Auramine-rhodomine stained stain and examined under FM, CY and PiLED florescence microscopes respectively. | 3 |

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| | 9 | Positive smears were graded as scanty if there were: 1-9 AFBs in at least 100 fields; +1: 10-99 in 100 fields; +2: 1-10 per field in at least 50 fields; and +3: if there were more than 10 per field in at least 20 fields. Absence of any AFB was graded as negative smear.<br>Cultures that indicated positive growth were removed from the MGIT machine and the presence of AFB confirmed by ZN smear microscopy. Those that do not indicate growth after 42 days were classified as negatives as recommended by the manufacturer and as was the standard practice. | 3-4 |
| | 10 | Three experts were involved with the execution and interpretation of the smear microscopy tests while two were responsible for the reference (culture) tests. All experts hold a minimum of bachelor's degree in microbiology and had worked for at least 2 years prior to the study conduct. | 4 |
| | 11 | Experts were blinded on the clinical diagnoses and outcome of the tests done for clinical care purposes. | 4 |
| *Statistical methods* | 12 | Diagnostic accuracies were measured using decision matrix tables and matched sample tables for positive and negative final outcomes as previously recommended for comparative analysis of diagnostic procedures performed on same samples (patients) by Hawass NE in Br J Radiol. 1997 Apr;70(832):360-6. Differences in tests performances together with their 95% confidence intervals were evaluated using the McNemar's test. | 4-5 |
| | 13 | Routine laboratory quality assurance measures were used to assess tests reliability, not reported in this manuscript. | 0 |
| RESULTS | | | |
| *Participants* | 14 | This study was conducted from July 3, to September 26, of 2009 | 3 |
| | 15 | Clinical and demographic information of participants was excluded to minimize bias and ensure complete de-identification of data | 3 |
| | 16 | Main inclusion criteria were clinical diagnoses of TB, patient not on anti-TB drugs at the time of the study and provision of three sputum samples. 205 patients with clinical TB were seen over the study period. 10 were on anti-TB drugs at presentation, 16 failed to provide any sputum sample, 10 provided between 1 to 2 samples while culture results of 19 patients were contaminated and therefore all were excluded from the analyses. | 3 |
| *Test results* | 17 | There was no TB treatment administered within the 24 hour interval in which samples were collected. The index and reference tests were performed on the same sample. | 3 |

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| | 18 | Disease severity was not graded in this study. However, there were patients with clinical diagnoses of pulmonary diseases other than tuberculosis. Most of those patients were not requested to provide sputum samples for tuberculosis test and such samples were not targets of this study. | 0 |
| | 19 | A cross tabulation of the results of the index tests by the results of the reference standard were provided in Tables 1 and 2. | 7-8 |
| | 20 | The tests were performed on sputum samples provided by patients, no intervention was involved and therefore no adverse events monitored. | 0 |
| *Estimates* | 21 | Details of the estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals) were provided in Table 4 of the manuscript. | 10 |
| | 22 | The outcomes of the index and reference standard test were binary (yes, or no). There were no outliers, no missing data. | 0 |
| | 23 | Variability of diagnostic accuracy between the smear microscopy readers was not evaluated, a smear was positive if AFBs were seen by all the three readers. | 4 |
| | 24 | Estimates of test reproducibility was not evaluated | 0 |
| DISCUSSION | 25 | The high sensitivity for AFB detection shown by the inexpensive light-emitting diode based Primo Star iLED florescence microscope in the hard operational conditions of a resource limited setting underscores the potentials for its scalability to remote clinics with similar operational conditions. This will increase TB detection and improve TB treatment uptakes in high burden settings with scarce resources. The optimal exposure duration for decontamination of sputum samples prior to examination and culture using the n-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) reagent in this setting is 15 minutes. | 13 |